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Genotoxicidade em humanos e espécies indicadoras expostas a resíduos de urânio

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Sónia Alexandra Leite Velho Mendo Barroso, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro, do Doutor Fernando José Mendes Gonçalves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro e da Doutora Ruth Maria de Oliveira Pereira, Professora Auxiliar Convidada do Departamento de Biologia da Faculdade de Ciências da Universidade do Porto.

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Basta um pouco de espírito aventureiro para estarmos sempre satisfeitos, pois nesta vida, nada sucede como desejávamos, como supúnhamos nem como tínhamos previsto.

Noel Clarasó

Dedico este trabalho ao meu marido e aos meus pais pelo incondicional apoio e por terem acreditado em mim

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palavras-chave

Urânio, minas, radionuclídeos, metais, genotoxicidade, biomarcadores, *Eisenia andrei*, *Apodemus sylvaticus*

resumo

Os riscos dos resíduos provenientes da extração do urânio estão associados ao seu conteúdo em metais e radionuclídeos, o que levanta preocupações às autoridades governamentais e à população em geral. As populações humanas e outras espécies animais que vivem em zonas de exploração de urânio poderão estar expostas à radiação através de resíduos e poeiras radioactivase também através de água e alimentos contaminados. A determinação dos riscos deste tipo de contaminantes é feita, principalmente, através da análise química de amostras ambientais, dando-se menos importância à determinação de efeitos biológicos. A determinação de efeitos biológicos causados pela exposição a poluentes, tem-se revelado muito importante para uma avaliação da qualidade ambiental, de modo a providenciar indicações acerca dos efeitos negativos nos seres vivos e também para complementar a informação dada pelas análises químicas de amostras ambientais. Este facto levou ao estabelecimento de biomarcadores, que consistem em respostas biológicas adversas que são específicas de uma exposição a toxinas ambientais, para serem usadas como ferramentas de avaliação da qualidade ambiental. Neste trabalho foram analisadas respostas a nível molecular e celular, em minhocas, ratinhos do campo e humanos, a fim de determinar o risco químico e radiológico dos resíduos provenientes da mina de urânio da Cunha Baixa. Este trabalho teve também como objectivo a clarificação das respostas subjacentes à exposição a metais e radionuclídeos, a fim de permitir o desenvolvimento de potenciais novos biomarcadores moleculares. Durante este trabalho foram efectuados ensaios com minhocas, em que estas foram expostas durante 56 dias a solo contaminado proveniente da mina de urânio da Cunha Baixa, em laboratório e *in situ*. Durante a exposição foram analisados vários parâmetros, como o crescimento, reprodução, bioacumulação de metais e radionuclídeos, histopatologia, danos no DNA, citotoxicidade e perfil de expressão genética. Para além disso, foram amostrados ratinhos do campo na mina de urânio da Cunha Baixa e numa área de referência para determinação de danos no DNA, níveis de expressão e mutações em genes supressores de tumores e também bioacumulação de metais. Por fim, foram recolhidas amostras de sangue em voluntários saudáveis pertencentes à população da aldeia da Cunha Baixa, para determinação de danos no DNA, imunofenotipagem e quantificação de metais no sangue. Os resultados revelaram que as minhocas assim como os ratinhos do campo foram negativamente afetados pela exposição aos resíduos mineiros em todos os níveis de organização biológica aqui analisados, o que faz destes organismos bons indicadores para a determinação do risco destas áreas contaminadas, evidenciando o potencial risco da exposição a estes contaminantes. Para além disso, o estudo feito à população da Cunha Baixa revelou danos no ADN e diminuição de populações importantes de células imunitárias (nomeadamente linfócitos T e NK), o que poderá resultar da exposição aos resíduos da mina, tornando as pessoas mais susceptíveis ao desenvolvimento de processos de carcinogénese. O presente estudo contribuiu significativamente para a caracterização dos riscos da exposição a resíduos provenientes de minas de urânio abandonadas, evidenciando os seus efeitos negativos a nível molecular e celular, que potencialmente poderão causar instabilidade genómica e aumentar o risco de desenvolvimento de doenças genéticas.

keywords **Uranium, mines, radionuclides, metals, genotoxicity, biomarkers, *Eisenia andrei*, *Apodemus sylvaticus*.**

abstract

The risks of uranium mill tailings are associated with their content in metals and radionuclides, raising concern among the general public and governmental authorities. Human and other living species from uranium mining districts may be exposed to radiation doses emitted by wastes; radioactive dust and contaminated water and foodstuffs. The determination of the risks of such hazards is usually carried out through chemical analysis of environmental samples, neglecting the assessment of its biological effects. The measurement of the biological effects of pollutants has become of major importance for the assessment of the quality of the environment, in order to provide indications of harmful effects on biota and to complement the information given by chemical analyses of environmental samples. Such scenario, have triggered the research to establish early warning signals of adverse biological responses to environmental toxins, named biomarkers, to be used as a tools for environmental quality assessment. In this work different exposure-related molecular and cellular responses were analyzed in earthworms, wood mice and humans, aiming for the determination of the chemical and radiological risk of the residues from the Cunha Baixa uranium mine, to indicator species and populations living nearbyand also to clarify underlying responses to metals and radionuclides exposure, pointing out for the development of potentially new molecular biomarkers.For that, earthworms were exposed for 56 days in the laboratory and *in situ* to contaminated soil from the Cunha baixa uranium mine and several parameters were assessed, like growth, reproduction, metals and radionuclides bioaccumulation, histopathology, DNA damages, cytotoxicity and gene expression profile. Wood mice were sampled in the Cunha Baixa uranium mining area and in a reference area to determine DNA damages, expression levels and mutations in tumour suppressor genes and also metals bioaccumulation. Blood samples were colleted from healthy individuals from the population living near the Cunha Baixa uranium mine, for DNA damages assessment, immunophenotyping and for the determination of the presence of trace elements. Results showed that earthworms and wood mice were negatively affected by the exposure to the uranium mining residues at all the biological levels of organization tested in this work, making them good indicator species for the determination of the risks of such contaminated areas, highlighting the potential risks associated with the exposure to these contaminants. Moreover, the study performed to the population of Cunha Baixa reveled DNA damages and depletion of important immune cell populations (mainly, T and NK lymphocytes), potentially due to the exposure to the radioactive mine residues, increasing the susceptibility for tumour growth.The present study contributed greatly for the characterization of the risks posed by the exposure to residues from abandoned uranium mines, highlighting the damaging effects at a molecular and cellular level, which potentially may cause genomic instability and increased risk of the organisms exposed to them, of developing genetic diseases

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Ap sites	Apurinic/apyrimidinic sites
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BAF	Bioaccumulation Factor
Bq	Becquerel
CAT	Catalase
CDK	Cyclin Dependent Kinase
cDNA	Complementary deoxyribonucleic acid
CR	Concentration Ratio
CRP	C-reactive Protein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double-strand break
E. andrei	*Eisenia andrei*
E. crypticus	*Enchytraeus crypticus*
EDTA	Ethylenediamine tetraacetic acid
ENU	Empresa Nacional Urânio
EPA	Environmental Protection Agency
EST	Expressed Sequenced Tags
FBS	Fetal Bovine Serum
ICRP	International Comission on Radiological Protection
IR	Ionizing Radiation
ISL	In Situ Leaching
ISO	International Standards Organization
LET	Linear Energy Transfer
MFI	Median Fluorescence Intensity
mRNA	Messenger Ribonucleic Acid
mtDNA	mitochondrial DNA
NK cells	Natural Killer cells

NRT	Non Reverse Transcriptase
NTC	Non Template Control
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate Buffered Saline
PCR	Polimerase Chain Reaction
Rb	Retinoblastoma gene
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
RT-qPCR	Reverse Transcriptase quantitative PCR
SNP	Single Nucleotide Polymorphism
SOD	Superoxide dismutase
SSB	Single-Strand Break
SSH	Suppression Subtractive Hybridization
UDL	Under Detection Limitis
UV	Ultra Violet radiation
WBC	Total White Blood Cells
WHC	Water Hold Capacity

CHAPTER I - GENERAL INTRODUCTION

1.1 Uranium mines

Anthropogenic sources of uranium include uranium mining and milling, uranium conversion and enrichment, uranium fuel fabrication, nuclear weapons production, production of phosphate fertilizers from phosphate rocks containing uranium, and the improper disposal of uranium mine tailings (ATSDR 2011).

The special risks associated with uranium mining became evident when workers appeared to be more susceptible to pulmonary disorders than workers from other mines (IAEA 2005a). Over the past decades these concerns for workers health and safety have been the main reasons for the development of improved practices and tighter controls (IAEA 2005a). Only recently, concerns over the impacts on public health and on the natural environment have been raised, regarding the full range of operational activities related to uranium mining and the rest of the nuclear fuel cycle (IAEA 2005a). As to the natural environment, these concerns include the risk of environmental degradation, contamination, reduced ecosystem viability and biodiversity, aesthetics, public amenities, access to land, and quarantine of land for future beneficial land use (IAEA 2005a; Antunes et al. 2007c; Antunes et al. 2008a; Pereira et al. 2008).

The legacies of past uranium mining and milling activities continue to be a cause of concern and require assessment and remedial action (IAEA 2005a). This problem has been recognized in many parts of the world over the past three decades, but has received special attention since the end of the Cold War (IAEA 2005a). The search for uranium covered almost all the countries in the world. The result in some countries is a legacy of numerous small scale mines and mills (IAEA 2005a). Due to economic stringencies, less demanding environmental legislations and lack of awareness at the time, these operations may have not been properly shut down and made safe from both a radiological and a general safety point of view (IAEA 2005a). Active and abandoned uranium mining sites can represent complex environmental situations, where health risks and environmental detriments may result from radon exhalation and dispersion of radioactive dust from mine wastes as well as from the discharge of contaminated mine waters into surface and groundwaters (IAEA 2005a; Carvalho et al. 2007a; Carvalho et al. 2009a; Carvalho et al. 2009b).

1.1.1 Uranium extraction

Uranium extraction can be performed by open pit mining, underground mining and *in situ* leaching (IAEA 2002). In conventional underground and open pit mining activities, economically

valuable ore is stockpiled and processed at the mill site, and the residual waste, mostly mill tailings, is normally disposed near the mill site (IAEA 2002). Ore mined in open pit or underground mines is crushed and leached in an uranium mill, a chemical plant designed to extract uranium from the ore (IAEA 2005a; Winfield et al. 2006). The mill tailings may also be subjected to heap leaching, where a lixiviant solution is distributed over the surface to extract the ore from the heap. Then the leachate is collected in a pond and sent to the mill for ore extraction (IAEA 2005a). *In situ* leaching (ISL) is an alternative method of extracting uranium. This process does not physically remove the host material from its underground location but preferentially extracts the uranium using an acid or alkaline solution, leaving the tailings in their original subterranean location (IAEA 2002). In this method, wells are drilled over an ore body, found in sandstone aquifers (IAEA 2005b). The water from these aquifers is extracted and treated with chemicals and pumped back into the aquifer, where the solution leaches the ore resulting in a concentrated solution of uranium and other elements (IAEA 2005b). The solution is pumped to the surface where the uranium is removed and then re-injected into the aquifer where the leaching cycle continues (IAEA 2005b). In all cases, the original host material is altered physically (crushing for conventional mining) and/or chemically (conventional milling and in situ leaching) to extract uranium (IAEA 2002). At each process phase, environmental media (e.g. air, surface water, groundwater) may interact with the material and potentially disperse contaminants to humans and environmental receptors (IAEA 2002, 2005a).

1.1.2 Mining and milling impacts

Usually, mines are extensively exploited and, even when the activities cease, the impacts onsite and offsite continue (Pereira et al. 2004b, c; Carvalho et al. 2007a; Carvalho et al. 2007b). At each stage of the mining and milling process, radioactive, hazardous and other wastes emissions and discharges are generated (Winfield et al. 2006). Both open pit mines and shaft mines create very large quantities of waste rock tailings, which are major sources of the biophysical impacts of uranium mining (IAEA 2002, 2005a; Winfield et al. 2006; Carvalho et al. 2007b). Tailings are the waste by-product of the milling process, and the amount produced is proportional to the grade and amount of the ore milled (IAEA 2002, 2005a; Winfield et al. 2006). Tailings consist of ground rock particles, water and chemicals used in the extraction process and contain radioactive and other non-radioactive hazardous constituents, namely radionuclides and

metals, respectively (IAEA 2005a; Winfield et al. 2006; Carvalho et al. 2007a). Up to 85% of the radiological elements, contained in the uranium ore end up in the tailings, namely: ^{210}Po, ^{210}Bi, ^{210}Pb, ^{222}Rn, ^{226}Ra, ^{230}Th, ^{234}U, ^{234}Th and ^{238}U (Winfield et al. 2006; Carvalho et al. 2007a; Carvalho et al. 2011). Radon gas is also released from the tailings, due to continuous production from the decay of ^{226}Ra, which has a half-life of 1.600 years, presenting itself as a long-term hazard (Winfield et al. 2006). Further, ^{226}Ra is also continuously produced by the decay of ^{230}Th (with a half-life of 80.000 years), which is also present in the tailings (Winfield et al. 2006). In addition to radionuclides, tailings may also contain metals (for example: nickel, cadmium, arsenic, selenium) and residual chemicals from the mill process (IAEA 2002, 2005a; Winfield et al. 2006). The tailings may also present a risk of acid drainage to surface and groundwaters as a result of the presence of sulphidic ore or chemicals introduced through milling (IAEA 2002, 2005a; Winfield et al. 2006). The windblown dust from tailing areas may also contribute for the release and spreading of these contaminants through the surrounding areas (IAEA 2005a; Winfield et al. 2006). Due to the presence of these kind of contaminants, uranium mill tailings need to be managed for an indefinite amount of time (Winfield et al. 2006). Dust containing metals and radionuclides can also be released, not only from tailings, but also from underground ventilation systems, waste rock, surface mining operations and milling operations (IAEA 2005a; Winfield et al. 2006). Radon, as well as uranium and ^{210}Pb can also be released from ventilation systems of underground mines and also from waste rock and ore storage areas (Winfield et al. 2006). Surface and groundwater may also be contaminated not only by acid drainage from tailings, but also through discharges of process and mine waters, leaching from waste rock and storage sites, general run-off from the mine sites and also by ISL extraction (IAEA 2005a, b; Winfield et al. 2006).

1.1.3 The case of the Cunha Baixa uranium mine

The mining of radioactive ores in Portugal started in 1912.The Rosmaneira mine was the first mining concession recorded (Carvalho et al. 2005a). Sixty mines were exploited for radioactive ore and the majority were located in Guarda, Viseu and Coimbra districts (Carvalho et al. 2005a). Most of them were of small size and exploited as open pits, however some of the largest ones were exploited as underground mines or a combination of both methods (Carvalho et al. 2005a). In most cases the uranium ore was extracted and then transported to chemical treatment facilities (Carvalho et al. 2005a). Some of the most important ones were located in the Viseu

district, namely: Urgeiriça (Canas de Senhorim), Quinta do Bispo and Cunha Baixa (Mangualde, Viseu) (Carvalho et al. 2005a).

The Cunha Baixa uranium mine is located approximately 20 km southeast of Viseu, near the Cunha Baixa village. Mining activities started in 1970 and ceased in 1993 (Neves et al. 2005). The underground and open pit mining works took place from 1970 until 1984 and produced about 1000t of U_3O_8 and one million tonnes of waste material that were disposed in a dump surrounding the mine area (Neves et al. 2005). From 1984 to 1993, the low-grade ores produced in Cunha Baixa and adjacent mines (500 000 t) were placed in the open pit area and submitted to heap leaching with sulfuric acid solutions, which created an artificial pond (Fig. 1)(Antunes et al. 2008a).

Figure 1: Artificial pond in the Cunha Baixa uranium mine

This pond is subjected to high water level variations, including drought, as a consequence of changes in the piezometric level of the aquifer (Neves et al. 2005; Antunes et al. 2008a). Sulfuric acid was also used for *in situ* leaching of uranium in the underground works (Carvalho et al. 2009a). The use of sulfuric acid as a leaching agent, as well as the nature of the geologic materials that compose the tailings, which have potential for acid drainage, give rise to acidic waters (Carvalho et al. 2005b; Pereira et al. 2008; Carvalho et al. 2009a). Seepage from acid mine waters and surface runoff from the tailings still impact surface waters and aquifers in the area (Carvalho

et al. 2005b). The mine pit water from the artificial pond, which remained after the heap leaching process, is acidic (pH<3.5) and therefore able to percolate through permeable heap leaching wastes, mobilizing elements and increasing their dispersion (Neves et al. 2005). Thus the open pit area also presents a risk to surface and groundwater by continuous contaminant leaching (Neves et al. 2005). These waters, which re-emerge in wells downstream, are unsuitable for human consumption, irrigation and livestock watering (Carvalho et al. 2005b; Neves et al. 2005), since they have low pH (<4.5) and high concentrations of metals and radionuclides (Carvalho et al. 2005b; Neves et al. 2005). Consumption of this water can expose humans living nearby and also domestic animals to radiation doses above the recommended doses and also to chemical toxicity from the dissolved metals (Carvalho et al. 2005b). The use of this water for irrigation also presents risks to soils (acidity and salinity hazards) and to crops (phytotoxicity), due to a high content, in the available soil fraction, of uranium (U) and other metals (Neves et al. 2005; Pereira et al. 2009), some them, like Co, Cu, Ba, Sr and Ni, have proved to be easily bioaccumulated by important crop species like lettuce (Pereira et al. 2009). Furthermore, the actual use of these waters for irrigation purposes may facilitate the transfer of radionuclides and metals through the food chain (Carvalho et al. 2005b).

Since cessation of mining, the acid water from the mine pit has been pumped out for neutralization and precipitation of radionuclides (Pereira et al. 2008; Carvalho et al. 2009a). In this process, pH is neutralized through the addition of burned lime, and radium and metals are removed by precipitation through the addition of barium chloride (Pereira et al. 2004a; Pereira et al. 2008; Carvalho et al. 2011). Then the water rests for some time in a settling basin (Fig. 2), for precipitation of the chemical compounds that gradually accumulate at the bottom (Pereira et al. 2004a; Carvalho et al. 2007a).

Figure 2: Water treatment settling basin in the Cunha Baixa uranium mine

The sludge (Fig. 3) that results from this process, is then removed and transported to the waste rock dump area or to the mine pit, whenever the maximum capacity of the basin is reached (Pereira et al. 2004a).

Figure 3: Sludge deposition area in the Cunha Baixa uranium mine

This sludge is composed by metals, radionuclides (mainly from the uranium series), hydroxides, sulfates and carbonates (Carvalho et al. 2007a; Pereira et al. 2008). Due to the high level of contamination of these residues, their deposition in these areas will contribute to the contamination of the soil compartment (mainly by U and its daughter radionuclides and metals such as Pb, Cd, Zn and Mn) posing serious risks to the populations that use these soils for agriculture, as well as to edaphic communities (Pereira et al., 2008).

The release of radioactive and toxic contaminants (e.g. ^{222}Rn, ^{226}Ra, metals) into the air, is also hazardous to local human populations and to the surrounding environment, even after the cessation of the mining activities (Neves et al. 2005). The detection of high concentrations of several radioactive and non-radioactive chemical compounds on samples collected in different environmental compartments around the Cunha Baixa mine, indicates that the dispersion of the contaminants associated with the operations in the mine, occurred (Pereira et al. 2004a; Pereira et al. 2008; Carvalho et al. 2009a).

According to the Portuguese government, Cunha Baixa was one of the uranium mines classified as requiring priority intervention (despacho conjunto nº 242/2002 ao abrigo do disposto alínea c) do artigo 2º do Decreto lei nª 198-a/2001, de 6 de Julho). Since September 2011, the mine has been subjected to a remediation process (Fig. 4) held by EDM (Empresa de Desenvolvimento Mineiro, SA), the Portuguese company responsible for the remediation and rehabilitation of former mining areas. The work that this company is developing aims to: i) reduce the efflux of contaminated water from the underground mine that is feeding the mine pit artificial pond; ii) to reduce the radioactivity at the surface by reducing the release of radon gas and by reducing the geochemical and hydrochemical dispersion of elements present in the tailings and in the sludge from the water treatment basin; iii) to optimize de processes of acid water treatments; iv) to improve the safety conditions for human populations and animals; v) to promote the irrigation of soils with good quality water and to treat the contaminated areas that are used for agriculture.

Figure 4: Remediation works in the Cunha baixa uranium mine (source: Dra. Ruth Pereira, photo taken in May 2012)

1.1.4 Major contaminants of uranium mining tailings

1.1.4.1 Metals

Metals occur as natural constituents of the earth crust, and are persistent environmental contaminants, since they cannot be degraded or destroyed (Duruibe et al. 2007). The anthropogenic sources of metals include former and present mining sites, foundries and smelters, combustion by-products and traffic (Duruibe et al. 2007). The generation of acidic waters, in mining sites like the Cunha Baixa uranium mine, is the main source of metal contamination, since they are able to mobilize elements from the mining tailings, increasing their dispersion and causing environmental damages (Lopes et al. 1999; Antunes et al. 2007b). Several elements were found on samples collected in different environmental compartments around this mine, among those were sulfates, fluorine, Ca, Fe, Mn, Al, S, Zn, Cu, Ni, Co, Be and U (Pereira et al. 2004a; Pereira et al. 2008). Acid mine drainage is generated when pyrite (FeS_2) and other sulfide minerals, present in mining sites, are exposed to air and water in the presence of oxidizing bacteria, such as *Thiobacillus ferrooxidans.* These microorganisms oxidize FeS_2 to produce metal ions and sulfuric acid (Duruibe et al. 2007). The sulfuric acid produced from this reaction will be able to dissolve metals present in the geologic material. Chemical speciation has an impact on

solubility, bioavailability, and persistence of metals and metal compounds in the environment and for some metals, speciation may influence the pattern of toxicity (Goyer et al. 2004). Solubility is one of the major factors influencing bioavailability and absorption of metals and metal compounds. The solubility of a metal compound depends on its chemical species, on the pH of the medium (H+ ions), and on the presence of other chemical species in the medium (Goyer et al. 2004). The toxicity of metals and their compounds largely depend on their bioavailability, i.e. the uptake mechanisms, intracellular distribution and the ability to bind to cellular macromolecules (Beyersmann and Hartwig 2008).

Uranium is the heaviest naturally occurring element existing in the earth crust (Barillet et al. 2010; ATSDR 2011). This element is present throughout the environment, in rocks, soil, air, plants, animals, surface and underground waters (Barillet et al. 2010). Natural uranium is a mixture of three isotopes, U-234 (^{234}U), U-235 (^{235}U) and U-238 (^{238}U), which are chemically the same, but have different radioactive properties (ATSDR 2011). There are three kinds of mixtures (based on the percentage of the composition of the three isotopes): natural, enriched and depleted uranium. In natural uranium, the most common isotope is ^{238}U, making up about 99.274%, followed by ^{235}U 0.720% and ^{234}U 0.005% (ATSDR 2011; Skwarzec et al. 2012). Enriched uranium suffered a process that increased the percentage of ^{235}U and ^{234}U in its composition, whereas depleted uranium is the portion that remains after the enrichment process, which consequently as lower levels of these two isotopes when compared to natural uranium (ATSDR 2011). Enriched uranium is classified by the percentage of ^{235}U. For nuclear energy purposes it typically contains 3% of ^{235}U, however when uranium is enriched for nuclear weapons it contains as much as 97.3% of ^{235}U (ATSDR 2011). ^{235}U is important for both nuclear energy and nuclear weapons, since it is the only isotope existing in nature that is fissile (can be broke apart by neutrons), producing a chain reaction, that releases high amounts of energy (Capannesi et al. 2010). Being a radioactive element, uranium undergoes spontaneous transformation (decay), in which energy is released (emitted) either in the form of particles, such as alpha or beta particles, or electromagnetic radiation with sufficient energy to cause ionization, such as gamma rays (ATSDR 2011). This decay results in the formation of different elements, and the radioactive ones will also decay. This process continues until a stable (non-radioactive) element is produced (ATSDR 2011).When a uranium isotope decays, it emits an alpha particle and is transformed into a radioactive isotope of another element (Fig.5) (ATSDR 2011; Skwarzec et al. 2012).

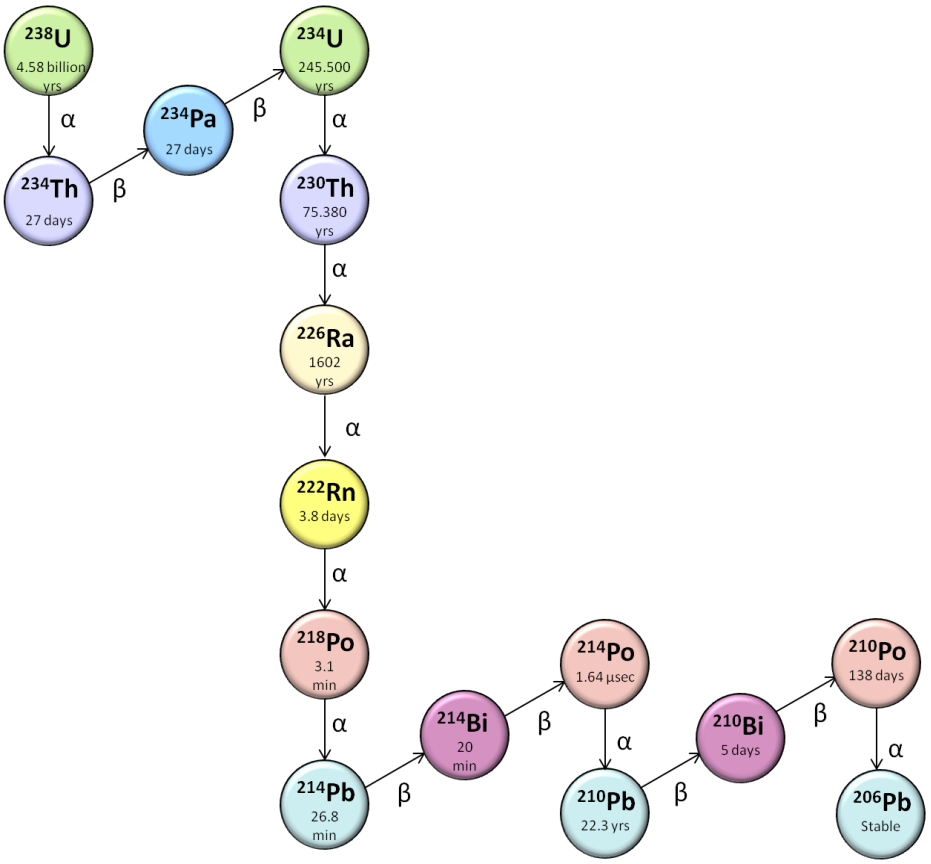

Figure 5: Uranium decay chain (adapted from www.gemoc.md.edu.au)

Metals exposure can cause genotoxicity, immunotoxicity, cytotoxicity, carcinogenicity, tissue damage, reduced growth and development, through different cellular pathways (Khalil et al. 1996; Wang and Shi 2001; Shrivastava et al. 2002; Leonard et al. 2004; Poleksic et al. 2010). The metal involved, the dose, the duration and the organs affected, and other environmental conditions, may all contribute to determining the pathway of cellular responses leading to toxic effects in the cells and ultimately to carcinogenesis (Leonard et al. 2004). Metals have the ability to induce toxicity by affecting pathways like DNA repair, regulation of nuclear transcription factors, gene expression regulation, apoptosis, cell growth regulation, metal-induced oncogene expression and effects on signal transduction pathways (Wang and Shi 2001; Leonard et al. 2004). Many metals, including carcinogenic metals, follow the metabolic pathways of similar essential metals (Goyer et al. 2004). This is probably the result of similar binding sites and affinities for carcinogenic metals and nutritionally essential metals, which is also a way of inducing toxic effects (Goyer et al. 2004). However, there are increasing evidences that reactive oxygen species (ROS)

generation play a major role in mediating metal-induced cellular responses and carcinogenesis and may also affect other pathways (Wang and Shi 2001; Leonard et al. 2004).

The role of ROS appears to be an important and nearly universally common step in metal-induced cellular responses (Fig. 6) (Ercal et al. 2001; Wang and Shi 2001; Leonard et al. 2004).

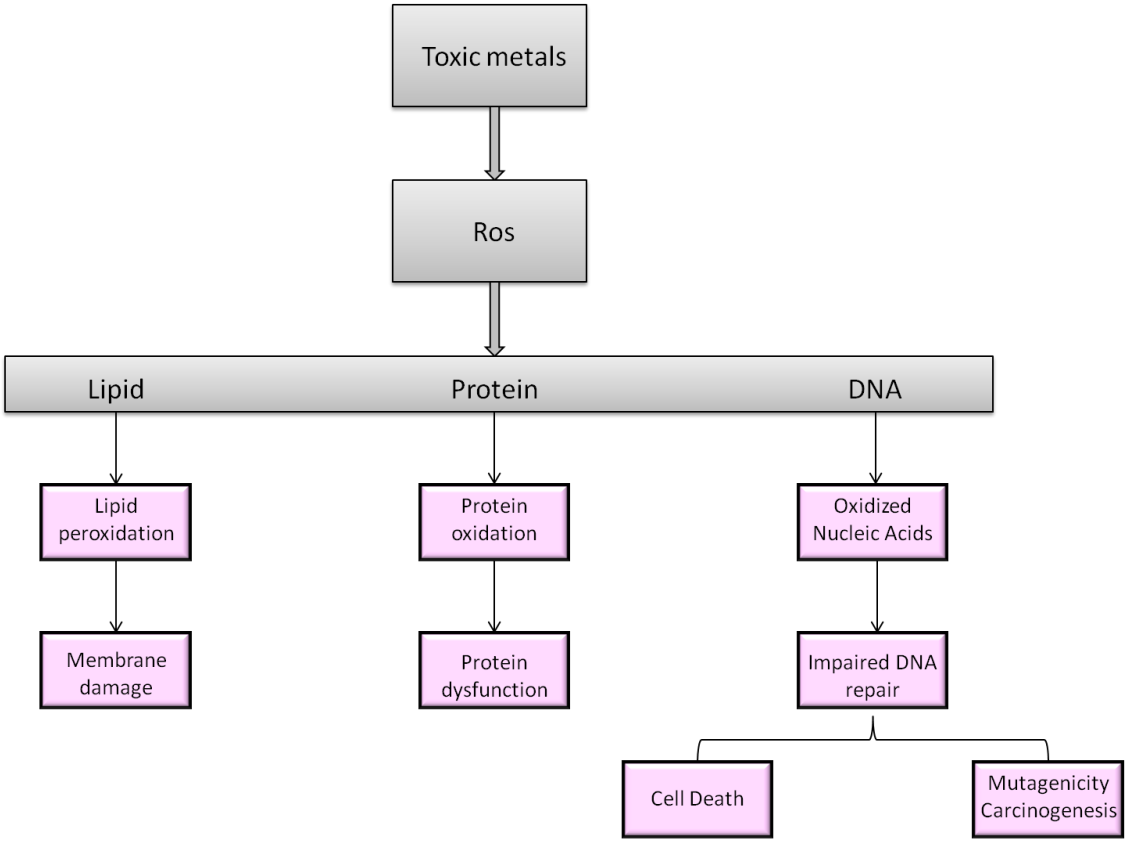

Figure 6: Consequences of metal induced oxidative stress (adapted from Ercal (2001))

These ROS include: superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxylradical (.OH), nitric oxide (NO.), peroxyl radical (ROO.), hypochloride (HOCl), alkoxyl radical (RO.) and thiyl radical (RS.) (Leonard et al. 2004). Whilst iron (Fe), copper (Cu), chromium (Cr), vanadium (V), cobalt (Co) and U (uranium) undergo redox-cycling reactions (Fig. 7) (e.g. Fenton reaction and Haber-Weiss cycle) , for a second group of metals, mercury (Hg), cadmium (Cd) and nickel (Ni), the primary route for their toxicity is depletion of glutathione and bonding to sulfhydryl groups of proteins (Ercal et al. 2001). A study performed by Yazzie et al. (2003) showed that apart from the generation of ROS, uranium can also damage DNA directly, through interaction between uranyl

cation and the negatively charged DNA phosphate backbone, thus resulting in DNA hydrolysis. Arsenic (As) is thought to bind directly to critical thiols, however, other mechanisms, involving formation of hydrogen peroxide under physiological conditions, have been proposed (Ercal et al. 2001).

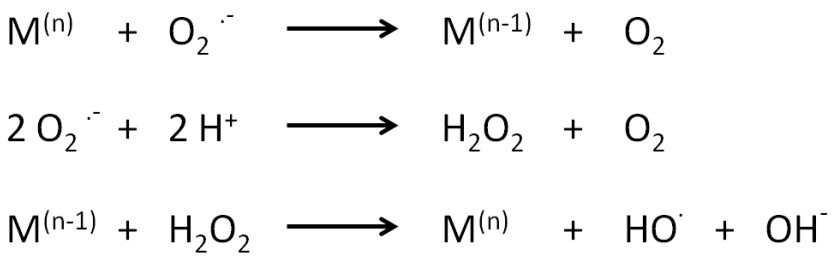

Figure 7: Fenton like reactions of metals (Ercal 2001)

Metal mediated formation of free radicals causes various modifications to DNA bases, enhanced lipid peroxidation, and altered calcium and sulfhydryl homeostasis (Ercal et al. 2001). Lipid peroxides, formed by the attack of radicals on polyunsaturated fatty acids residues of phospholipids, can further react with redox metals, producing mutagenic and carcinogenic malondialdehyde, 4-hydroxynonenal and other exocyclic DNA adducts (Ercal et al. 2001).

1.1.4.2 Radionuclides

Uranium minerals are always associated with other radioactive elements that arise from the radioactive decay of uranium, as a result uranium production generates waste containing radioactivity that may eventually be dispersed in the environment and become of radiological concern (Carvalho and Oliveira 2007). After the closure of the mining activities, particularly at those places were the ore was leached with sulfuric acid, the solid waste has been stocked as tailings that still have radioactive materials, containing several types of radionuclides (Carvalho and Oliveira 2007).

A nuclide is a general term applied to all atomic forms of an element (Luig et al. 2000). Nuclides are characterized by the number of protons and neutrons in the nucleus, as well as by the amount of energy contained within the atom (Luig et al. 2000). A radionuclide is an atom with an unstable nucleus, which is characterized by excess energy available to be imparted either to a

newly created particle within the nucleus or to an atomic electron (Luig et al. 2000). In this process, the radionuclide undergoes radioactive decay, and emits subatomic particles (α and β particles) and/or gamma rays, which, along with X-rays and high-frequency ultraviolet radiation, constitutes the ionizing radiation (Luig et al. 2000). An alpha particle (α) is a positively charged helium nucleus emitted by a larger unstable nucleus (Fig. 8) (IAEA 2004). It is a relatively large particle that consequently, has a short range through the air (1-2 cm) and can be completely stopped by paper or skin (Fig. 8) (IAEA 2004). Alpha radiation can, however, cause serious damages if it enters the body by inhalation or ingestion, due to its great potential for damaging DNA (IAEA 2004). A beta particle (β) is an electron emitted by an unstable nucleus (Fig. 8). Being much smaller than alpha particles, it has the ability to penetrate further into materials or tissue (Fig. 8) (IAEA 2004). Beta radiation can be stopped completely by sheets of plastic, glass or metal and normally it does not penetrate beyond the top layer of skin (Fig. 8) (IAEA 2004). However, large exposures to high-energy beta emitters can cause skin burns and can also be hazardous if inhaled or ingested (IAEA 2004). Gamma radiation (γ) is a form of electromagnetic radiation (Fig. 8), composed by photons, emitted by an unstable nucleus that is often emitting beta radiation simultaneously (IAEA 2004).

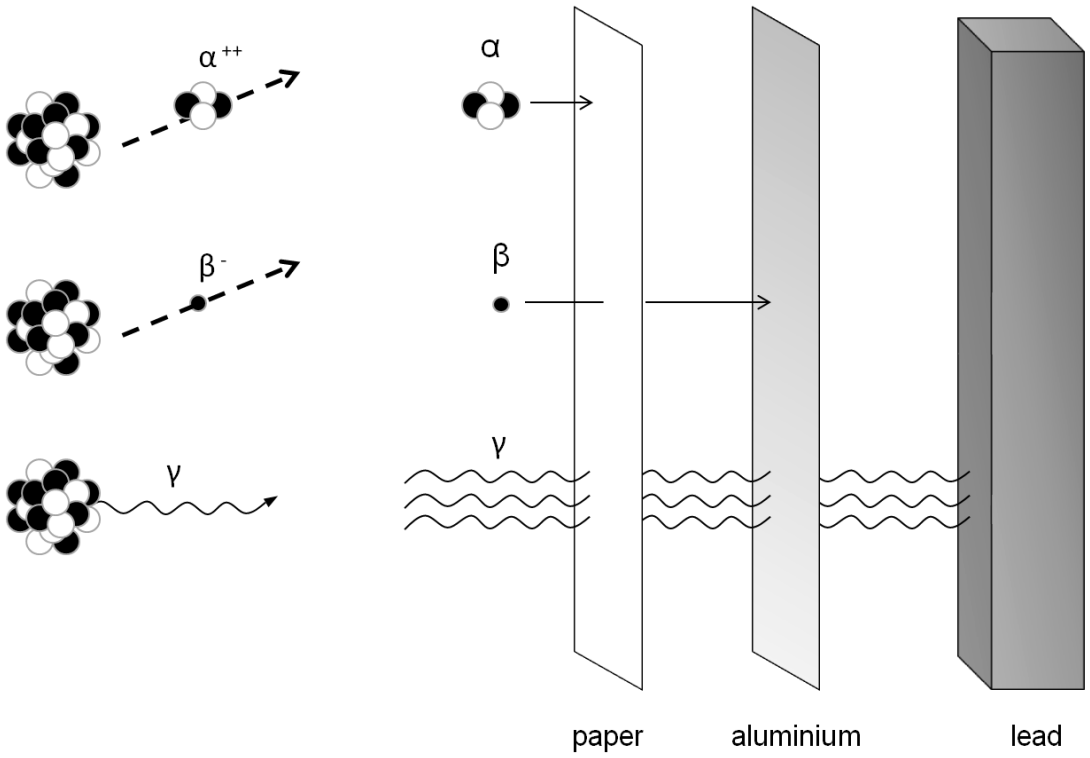

Figure 8: Alpha, beta and gamma radiation and their penetrating capacity (adapted from radiology.rsna.org and news.bbc.co.uk).

Gamma radiation causes ionization in atoms as it traverses matter, primarily due to interactions with electrons (IAEA 2004). It can be very penetrating and only a substantial thickness of dense materials such as steel or lead can provide good shielding (Fig. 8) (IAEA 2004). Gamma radiation can therefore be hazardous to internal organs without inhalation or ingestion (IAEA 2004).

Different types of radiation are known to vary in their effectiveness in causing damages to the cells and cancer (UNSCEAR 2000; Valentin 2003; Harrison and Day 2008). These differences can be related to the three-dimensional structure of ionization tracks produced by charged particles traversing tissue, containing sensitive cellular targets including chromosomal DNA (Harrison and Day 2008). In order to deduce the extent of the possible biological effects, a simple one-dimensional indicator of track structure, called linear energy transfer or LET, is used (UNSCEAR 2000; Harrison and Day 2008). There are extensive radiobiological data indicating that high-LET radiations (neutrons and alpha particles) have a greater biological effect, per unit of average absorbed dose, than low LET radiation (beta particles and gamma rays) (UNSCEAR 2000).

The most significant radionuclides present on samples collected in the different compartments (soil and water) around the Cunha Baixa mine, are for instance those from the Uranium-chain like ^{238}U, ^{226}Ra, ^{234}U, ^{230}Th, ^{210}Po and ^{210}Pb (Pereira et al. 2004a; Carvalho et al. 2009a; Lourenço et al. 2012). The majority of these radionuclides are alpha emitters, which if inhaled or ingested, increase the risk of severe damage to cells and consequently cancer, due to the emission of high LET radiation (UNSCEAR 2000, 2006). The *in situ* leaching technique applied in this mine, acidified the water from rivers and aquifers, increasing the levels of these contaminants, namely ^{238}U and ^{226}Ra, in wells used by the population living nearby (Carvalho et al. 2005b; Carvalho et al. 2009a) . Since the wells are used for irrigation, this will facilitate transfer of these radionuclides through the food chain (Carvalho et al. 2005b). Also the sludge from the settling basin used for the treatment of the mine pit waste water is heavily contaminated by radionuclides from the uranium series (Carvalho et al. 2007a). When this sludge is removed and spread near the mine pit it enhances the contamination of soils (Pereira et al. 2008).

1.2 Sub lethal effects

1.2.1 Genotoxicity

Genotoxicity is a deleterious effect caused by chemical compounds and certain types of radiation, on a cell's genetic material, thus affecting its integrity. Genotoxic substances interact and modify DNA´s structure and composition, which renders them mutagenic and potentially carcinogenic. The exposure to toxic chemicals, like metals and radionuclides, may generate DNA lesions such as adducts, single and double-strand breaks, base modifications and base loss (with the production of apurinic and apyrimidinic sites), DNA-DNA crosslinks and DNA-protein crosslinks (Fig. 9) (Shugart 2000; Barillet et al. 2010).

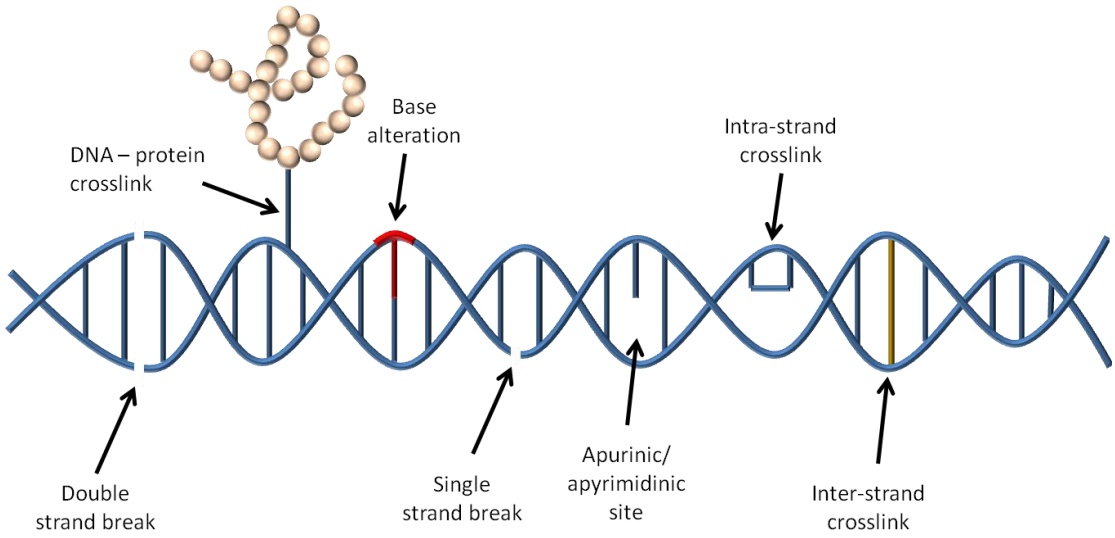

Figure 9: Examples of DNA damages caused by genotoxic substances (adapted from www.ilo.org).

Metals may be carcinogenic in the form of free ions, metal complexes, or particles of metals associated to poorly soluble compounds (Beyersmann and Hartwig 2008). One determinant factor in metal carcinogenesis is the bioavailability of different metal species, to which the cell membrane serve as an important barrier to its entrance into the cell (Goyer et al. 2004; Beyersmann and Hartwig 2008). In most cases, one inside the cell, DNA does not seem to be the primary binding site for carcinogenic metal ions (Beyersmann and Hartwig 2008). Even though, they can form adducts with DNA bases, due to their cationic character, interactions with proteins seem to be the preferred pathway inside intact cells (Beyersmann and Hartwig 2008). One

important exception is chromium (VI), since after its intracellular reduction to chromium (III) it binds readily to DNA, forming DNA-protein and DNA-DNA crosslinks (Beyersmann and Hartwig 2008).

The induction of oxidative DNA damage has been observed for most metals, and common mechanisms include the interference with antioxidant defense systems, and/or the catalysis of Fenton-type reactions, where endogenously formed ROS like hydrogen peroxide is converted into far more reactive hydroxyl radicals (Ercal et al. 2001; Wang and Shi 2001; Leonard et al. 2004; Beyersmann and Hartwig 2008). Also, ROS may be generated in the course of intracellular reduction of metals, like for example, the reduction of chromium (VI) to chromium (III) (Ercal et al. 2001; Beyersmann and Hartwig 2008). Besides generating DNA damage directly, low levels of ROS function as mitogenic signals and activate redox sensitive transcription factors (Beyersmann and Hartwig 2008), which may deregulate cell growth and promote tumour formation (Beyersmann and Hartwig 2008). Additionally, DNA repair mechanisms are frequent targets for disturbance by toxic metals (Leonard et al. 2004). Inhibition of repair and persistent DNA damage results in genomic instability (Beyersmann and Hartwig 2008) and may contribute to increase the mutagenicity of other genotoxic agents and also to increase endogenous mutation rates (Beyersmann and Hartwig 2008). DNA repair systems not only provide high protection towards environmental mutagens, but also to endogenous DNA damage occurring continuously (for example, due to oxygen metabolism) (Hartwig and Schwerdtle 2002; Leonard et al. 2004; Beyersmann and Hartwig 2008). Carcinogenic metals can also alter cell growth, either by affecting the expression of growth stimulating factors (inducing the expression of proto-oncogenes), or inactivating growth control mechanisms, by inactivating/downregulating the expression of tumour suppressor proteins (Beyersmann and Hartwig 2008). Finally, metal ions may also deregulate cell proliferation by inactivating apoptotic processes (Beyersmann and Hartwig 2008).

Chromossomal DNA damage, is considered to be the key event by which radiation induce the development of cancer and hereditary disease (UNSCEAR 2000). Radiation affects DNA either through direct interaction of ionizing particles with the DNA molecule or through the action of free radicals or other chemical intermediates produced by the interaction of radiation with neighboring molecules (UNSCEAR 2000; Barillet et al. 2010; Kiang et al. 2010). The degree of damage is proportional to the absorbed dose of radiation, and also depends on the type of radiation, since high and low LET ionizing radiation produce different types of DNA damages (UNSCEAR 2000; Harrison and Day 2008; Kiang et al. 2010). While the highly penetrating low LET ionizing radiation (beta particles, gamma rays), transfer their energy as waves with lower

ionization capacity, thus causing DNA damage mostly indirectly via de formation of free radicals, damages caused by high LET radiation (neutrons, alpha particles, with high ionization capacity) are more complex and difficult to repair, since it comprises closely spaced DNA damages (Hada and Georgakilas 2008; Kiang et al. 2010). Random energy deposition by ionizing radiation induces a wide variety of DNA lesions like single (SSBs) and double strand breaks (DSBs), oxidized bases and apurinic-apyrimidinic (abasic, AP) sites (Little 2000; UNSCEAR 2000; Hada and Georgakilas 2008). In addition to direct breaks induced by radiation, some post-irradiation breaks can be also formed as a result of the attempt to repair lesions such as AP sites, which can later be converted to SSBs or DSBs (Hada and Georgakilas 2008; Magnander and Elmroth 2012).Two or more DNA lesions of the same or different nature may be produced in close proximity to each other on the same or on opposite DNA strands (bistranded lesions) (Hada and Georgakilas 2008; Magnander and Elmroth 2012). These closely spaced (within 10-20 base pairs) types of DNA damage are referred as clustered DNA damages (Hada and Georgakilas 2008; Magnander and Elmroth 2012). Clustered DNA damages, together with the complexity of the damage, has been shown to increase with LET (Harrison and Day 2008; Kiang et al. 2010). While isolated damages are generally repaired efficiently, clustered DNA lesions are more difficult to repair, being generally considered repair-resistant or non-repairable, and have been reported has having a high mutagenic potential (Hada and Georgakilas 2008; Harrison and Day 2008). Cytotoxic effects of ionizing radiation are thought to result mainly from incompletely or incorrectly repaired DNA lesions (Hada and Georgakilas 2008).

Carcinogenesis is initiated in cells where critical biochemical sites are damaged. These sites include tumour suppressor genes that may be affected by serious damages to their nucleotide sequences. These damages may cause their inactivation or inappropriate expression, providing a mechanism for radiation induced malignant transformation (Little 2000).

1.2.1.1 Tumor-suppressor genes

Tumour suppressor genes encode proteins that inhibit cell transformation and whose inactivation, is therefore, advantageous for tumour cell growth and survival (Osborne et al., 2004; Oliveira et al., 2005; Hayslip and Montero, 2006). Tumour suppressor genes participate in a variety of critical and highly conserved cell functions, which include, regulation of the cell cycle, apoptosis, differentiation, surveillance of genomic integrity and repair of DNA errors, signal transduction and cell adhesion (Oliveira et al., 2005). Consequently, they prevent deregulated

progression through the cell cycle, induce apoptosis in cells with unrepairable DNA damages and inhibit cellular migration and metastasis (Hayslip and Montero, 2006).

Conceptually, tumour suppressor functions can be separated into two major categories, depending on whether the functions are viewed as gatekeeper or caretaker roles (Van Heemst et al., 2007). Gatekeepers, directly inhibit tumour growth or promote tumour death and their inhibition will contribute directly to cancer formation and progression (Van Heemst et al., 2007). Caretakers are genes whose loss of function is not directly responsible for tumour development; however their inactivation will result in increased occurrence of genomic mutations, which may inactivate gatekeeper tumour suppressors (Van Heemst et al., 2007). Examples of gatekeeper tumour suppressors are the *p53* and *rb* genes (Oliveira et al., 2005). These genes are conserved from invertebrates to mammals, namely worms, flies and vertebrates (Hu, 2009; Cao et al., 2010). Tumour suppressor genes may lose their functions through mutations, deletions, epigenetic events such as promoter methylation, inappropriate expression of transcriptional repressors, and loss of transcriptional activators (Oliveira et al., 2005; Hayslip and Montero, 2006).

The alteration and disturbance of DNA's structure and composition, may lead to the activation or inactivation of tumour suppressor genes, either through mutations or deregulation of their expression patterns. Previous investigations showed that the exposure to metals, such as Cr, Ni, As, Cd, V and Pb, is able to activate cellular transcription factors, like *p53*, in response to exposure (Harris and Shi, 2003; Leonard et al., 2004). Some chemical form of metals, like As(III) and Cr(VI), are able to induce the expression of *p53*, leading to apoptosis of the affected cells (Harris and Shi, 2003; Pulido and Parrish, 2003; Leonard et al., 2004). Others, like iron (in hemachromatosis) and nickel, are able to induce mutations in the *p53* gene and to affect its overexpression (Harris and Shi, 2003). The overexpression of *p53* is also observed in many forms of cancer induced by metal exposure (Harris and Shi, 2003). Mutations in the *p53* gene, as a consequence of exposure to alpha particles, in uranium mining workers, were also reported (Hollstein et al., 1997). Radiation has also been shown to increase *p53* levels in response to DNA damage (Little, 2000). It has been proposed that the most likely mutational event in the initiation of radiation carcinogenesis involves loss of heterozygosity of a tumour suppressor gene (Kim and Lee, 2003). Although few studies were carried out, *rb* gene was shown to lose heterozygosity in patients with retinoblastoma, treated with radiotherapy (Little, 2000; Kim and Lee, 2003). The loss of heterozygosity in a cell implies the inactivation, through mutational events, of one allele of a gene in which the other allele was already inactivated. Radiation has also shown to affect the expression of *rb* gene(Gobé et al., 1997). Since alterations in the *p53* and *rb* genes are frequently

associated with the development of many cancers, they may be considered as early warning signal of genomic instability.

1.2.1.1.1 *p53* tumor suppressor gene

The human *p53* belongs to a highly conserved gene family (Bai and Zhu, 2006). It is a nuclear phosphoprotein of 53 kDa, encoded by a 20 kb gene, containing 11 exons, that is located on the small arm of chromosome 17 (Bai and Zhu, 2006).

The P53 tumour suppressor protein plays a pivotal role in the cellular response to a range of environmental and intracellular stresses (Meek, 2004). The P53 protein is a multifunctional transcription factor that regulates the expression of other genes involved in cell cycle control, apoptosis, DNA repair and angiogenesis (Kishore et al., 2007). Its primary role is to act as a transcription factor to induce the expression of proteins involved in cell cycle arrest and apoptosis (Coates et al., 2005). P53 is involved in the control of the G1/S cell cycle transition, by enhancing the transcription of the cell cycle inhibitor p21waf/CIP that exerts a negative effect on the cyclin-dependent kinase complex (CDK) (Meek, 2004; Coates et al., 2005; Oliveira et al., 2005). Inhibition of CDK then blocks cell cycle progression by activating the Rb tumour suppressor and by inhibiting the expression of cyclin D1 (Meek, 2004; Oliveira et al., 2005). P53 can also regulate G2/M transition, which is accomplished by the inhibition of Cdc2 and stimulating the transcription of cell cycle inhibitors (Meek, 2004; Oliveira et al., 2005). Because of the short half-life of the P53 protein, its activity is maintained at low levels in the absence of stress (Lee et al., 2007), by being constantly produced and degraded through ubiquitination by Mdm-2 (Coates et al., 2005). Upon exposure to a variety of stress conditions, the stability of P53 increases and the protein accumulates in the nucleus, were it is activated (Lee et al., 2007). The stabilization and activation of P53 is largely mediated through posttranslational modifications, such as phosphorylation and acetylation, as well as through protein-protein interactions (Lee et al., 2007). The pattern of phospohorylation after genotoxic damage has been known to vary significantly with the genotoxic agent and also with the dose to which cells are exposed to (Coates et al., 2005). P53 influence DNA repair through transcriptional mechanisms and direct interaction with repair proteins (Coates et al., 2005). When DNA repair is not possible, P53 induce the expression of proteins involved in apoptosis (Coates et al., 2005), in order to inhibit the formation and growth of tumour cells . Induction of *p53* can occur in response to a range of genotoxic stresses leading to the

biological outcomes of growth arrest or apoptosis (Meek, 2004). It has been reported that the *p53* gene is deleted or mutated in up to 50% of all cancers (Gasco et al., 2002).

1.2.1.1.2 Retinoblastoma (*rb*) tumor suppressor gene

The human *rb* gene is located on 13q14 chromossome and is composed by 27 exons , encoding a 110 kDa protein, that can be detected in all human tissues (Dick, 2007). Among other cellular functions, it has a key role in cell cycle check point control, particularly in the transition from the G1 to the S phase of the cell cycle (Zheng and Lee, 2001; Oliveira et al., 2005; Dick, 2007). Rb protein alternates between hypophospohorylated (active) and hyperphosphorylated (inactive) forms, depending on the phosphorylation events from upstream signalling pathways (Oliveira et al., 2005; Dick, 2007; Henley and Dick, 2012). When the Rb protein is active, it blocks cell cycle progression by binding and inactivating transcription factor E2F, which controls the expression of target genes necessary to proceed to the S phase (Henley and Dick, 2012). During the progression of the cell cycle, Rb is gradually phosphorylated and, consequently inactivated by cyclin dependent kinases (CDK) complexes, causing Rb to release E2F factor (Dick, 2007; Henley and Dick, 2012). The inactivation of *rb* gene may result from intragenic mutation, chromosomal deletion, promoter methylation, transcriptional silencing and/or functional inactivation (Oesterreich and Fuqua, 1999; Oliveira et al., 2005). The inactivation of this gene, leads to the disruption of the mitogenic factor E2F regulation, which will stimulate the abnormal cell proliferation (Oesterreich and Fuqua, 1999; Oliveira et al., 2005; Dick, 2007). In addition to its role as a cell cycle gatekeeper, Rb protein has also other important roles such as the control of cellular differentiation, regulation of cell apoptosis and preservation of chromosomal integrity, by protecting cells from double-strand breaks (DSBs) that arise during DNA replication or after DNA damage (Zheng and Lee, 2001; Genovese et al., 2006; Dick, 2007). Rb is crucial for the maintenance of the DNA damage checkpoint (G1/S) function, because it elicits cell cycle arrest in response to a variety of genotoxic stresses (Genovese et al., 2006). The three facets of Rb action in the DNA damage checkpoint response can be divided into (i) transcriptional repression of E2F-regulated genes; (ii) induction of cell cycle arrest; (iii) inhibition of DNA DSBs accumulation (Genovese et al., 2006).

1.2.2 Immunotoxicity

Immunotoxicity can be defined as any adverse effect on the immune system that can result from the exposure to a range of environmental agents including chemicals. Among the alterations identified in physiological systems, chemically induced immunological disorders have been well documented in an increasing number of species (Auffret et al. 2006).

Several classes of xenobiotics may induce severe alterations on both structure and function of the immune system, even at low concentrations (Auffret et al. 2006). The exposure to metals like, cadmium (Cd), lead (Pb) and nickel (Ni), can modulate the immune response of a variety of animal species, including humans, at exposure levels below which, other more commonly used biomarkers of toxicity do not respond (Zelikoff 1998). The metal generated changes in protein activity, that modulate signaling pathways, as well as changes in the expression of genes which are able to regulate the synthesis of protein molecules such as cytokines and their receptors and surface adhesion molecules, predominate in the immunotoxic effects (Lutz and Wasowicz 2003). The immune system is characterized by high precision autoregulatory mechanisms, controlled by different populations of cells and cellular mediators (Lutz and Wasowicz 2003). These precise mechanisms may be seriously disturbed by the effects of chemicals present in the environment (Lutz and Wasowicz 2003). Metals may disrupt immune homeostasis by modulating immunoregulatory activities and leading to either immunoenhancement or immunosuppression (Lawrence and McCabe Jr 2002). Nonetheless, the extent of these effects varies with the type of metal, its concentration, biologic availability and a number of other factors. (Lawrence and McCabe Jr 2002). Metals may disrupt the production of cytokines, their receptors, and adhesion molecules at the cellular genome and protein synthesis levels (Lutz and Wasowicz 2003). Insufficient activation or inhibition of these proteins synthesis leads to decreased immunity (immunosuppression), whereas its overactivation induces hypersensitivity (allergy, autoimmunization) (Lutz and Wasowicz 2003). Immunosuppresion is associated with decreased humoral and cellular responses, by compromised lymphocyte proliferation and decreased thymic weight (Fournier et al. 2000), which will probably lead to decreased host resistance against challenges by infectious agents or tumour cells (Zelikoff 1998). Metals can cause inadequate or excessive production of inflammatory cytokines and also inappropriate activation of lymphoid subsets, involved in acquired immunity, to specific antigens, resulting in pathologies such as chronic inflammatory processes and autoimmune diseases (Lawrence and McCabe Jr 2002). Lutz

et al. (2003) also refer that the activation of proteins involved in cellular signaling and the activation of expression of genes regulating the synthesis of proteins participating in the immune response, are associated with oxidative metabolism of immune cells and that metals modulation of the immune response influences the mechanisms that control the production of ROS.

It has been known that the hematopoietic system is highly sensitive to uranium and to ionizing radiation, which is associated with an increased risk of cancer development (Kiang et al. 2010; Wagner et al. 2010; Bao et al. 2012). Immunosuppression is a consequence of whole body irradiation at medium to high doses, and can also be a consequence of localized radiotherapy (UNSCEAR 2006b). In contrast, it has been reported that very low doses of ionizing radiation may give rise to immunostimulatory effects, particularly at short times after irradiation (UNSCEAR 2006b). Because of these divergent effects, ionizing radiation is probably better considered as an immunomodulatory rather than as an immunosuppressive agent (UNSCEAR 2006b). The studies reported in UNSCEAR(UNSCEAR 2006b) on the effects of low dose/low dose rate irradiation exposure, showed that: i) hematopietic stem cells and blood cell progenitors seem to be the main target; ii) low but significant suppression of blood leucocytes, including granulocytes, monocytes and lymphocytes; iii) direct irradiation damage to the blood/immune precursor pool results in decrease of stem cell fraction; iv) decreased viability of mature blood cells results from ineffective haematopoiesis, thus causing restriction of myeloid (and probably lymphoid) cell reserves; v) disturbances of cellular and humoral activity are likely to be caused by extreme radiosensitivity of lymphoid tissues and by a restricted progenitor cell pool.

1.2.3 Histopathology

As above referred, one of the major mechanisms of metal induced toxicity has been attributed to oxidative stress. Studies have shown that the generation of reactive radicals, result in cellular damage like depletion of enzyme activities, damage to lipid bilayer and DNA. These reactive radical species include a wide variety of oxygen-, carbon-, sulfur- and nitrogen- radicals, originating not only from superoxide radical, hydrogen peroxide, and lipid peroxides but also in chelates of amino-acids, peptides, and proteins complexes with the toxic metals (Flora et al. 2008). Reactive species generated as a consequence of metal exposure, may cause neurotoxicity, hepatotoxicity and nephrotoxicity in humans and animals (Flora et al. 2008). ROS can also act indirectly by modifying the cellular redox potential, which modulates key regulatory proteins

involved in programmed cell death (Azevedo et al. 2009).Programmed cell death, in which cells actively participate in their own death, is characterized by a pattern of structural changes in the nucleus and cytoplasm, including rapid blebbing of the plasma membrane, DNA fragmentation and formation of membrane-enclosed cell fragments (apoptotic bodies)(Azevedo et al. 2009). Metals may also induce necrosis, which is characterized by loss of control of ionic balance, swelling and cell lysis (Campbell et al. 2010). As a consequence of the lysis process, many intracellular constituents are released, attracting immune cells and causing inflammatory responses (Campbell et al. 2010). Ionizing radiation exposure also leads to increases in reactive oxygen/nitrogen reactive species that cause alterations in cell function/phenotype, resulting in chronic inflammation, organ dysfunction and ultimate fibrosis and/or necrosis (Zhao et al. 2007). Evidences suggest that the development and progression of radiation induced late effects are also driven, in part, by oxidative stress.

1.3 Biomarkers

In recent years, as a consequence of anthropogenic activities, the concern about the levels of contaminants and the risks they pose has increased. Consequently, the measurement of the biological effects of pollutants has become of major importance for the assessment of the environmental quality, in order to provide indications of the harmful effects on biota and to complement the information given by chemical analyses of environmental samples (Cajaraville et al. 2000; Lam and Gray 2003; Van Der Oost et al. 2003). Such a scenario has triggered research to establish early warning signals of adverse biological responses to environmental toxins, designated biomarkers, to be used as tools for environmental quality assessment (Cajaraville et al. 2000; Van Der Oost et al. 2003).

Depledge et al. (1994) defined “biomarker” as biochemical, cellular, physiological or behavioral variations that can be measured in tissue or body fluid samples, to provide evidence of exposure and/or effects from one or more contaminants. Others, defined biomarker as any biological response to an environmental chemical at the sub-individual level, measured inside an organism or in its biological products (urine, faeces, hair, feathers, etc), indicating a deviation from the normal status that cannot be detected in the unexposed organism (Van Der Oost et al. 2003). The term biomarkers is generally limited to sub-individual changes, since one of the functions of biomarkers is to provide early warning signals of biological effects, which are believed to precede those that occur at higher levels of biological organization (Fig. 10)(Lam and Gray 2003).

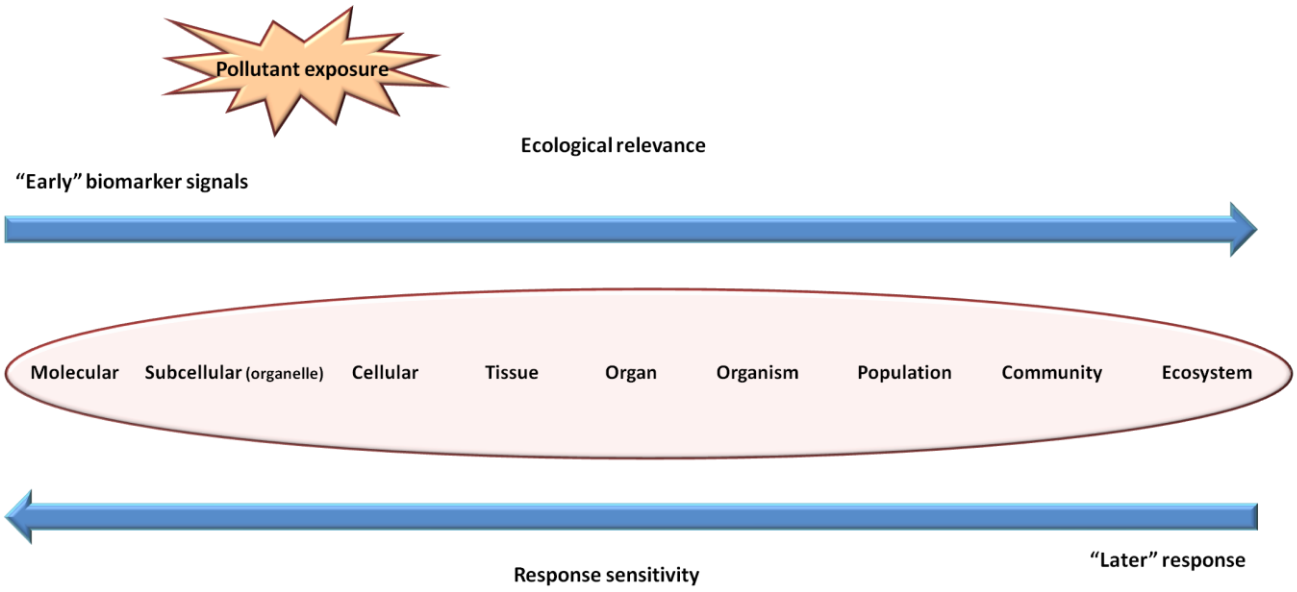

Figure 10: Sequential order of responses to pollutant exposure within a biological system (adapted from Van der Oost et al., 2003).

Generally speaking, biomarkers may be classified into three types: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility (Lam and Gray 2003; Van Der Oost et al. 2003; Hagger et al. 2006). Biomarkers of exposure indicate that an organism has experienced exposure to a toxicant or other stressor, since they cover the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism, however it is not predictive of the degree of adverse effects (Van Der Oost et al. 2003; Hagger et al. 2006). Biomarkers of effect are associated specifically with the toxicant mechanism of action and relate the degree of biomarker modification to the degree of adverse effects (Lam and Gray 2003; Van Der Oost et al. 2003; Hagger et al. 2006). Biomarkers of susceptibility, indicate the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic (Van Der Oost et al. 2003). However, the subdivision of biomarkers in the literature is not clear; since biomarkers of exposure and those of effect are distinguished by the way they are used, not by an inherent dichotomy (Suter 1993; Van Der Oost et al. 2003). The response of biomarkers can be regarded as biological or biochemical effects after a certain toxicant exposure, which makes them theoretically useful as indicators of both exposure and effects (Van Der Oost et al. 2003).

The use of biological markers or biomarkers measured at the molecular or cellular levels have been proposed as sensitive tools for biological effect measurement for environmental quality

assessment (Cajaraville et al. 2000). Thus these “early warning” biomarkers can be predictive, since they have the potential to anticipate changes at higher levels of biological organization (population, community or ecosystem) (Fig. 9), allowing mitigation actions before irreversible damages occur (Cajaraville et al. 2000; Van Der Oost et al. 2003). Biomarkers are thus defined as short-term indicators of long-term biological effects (Cajaraville et al. 2000).

Despite the definition of biomarkers of exposure, which include the detection and measurement of an exogenous substance within an organism, body burdens are not considered to be biomarkers or bioindicators, since they do not provide information related to “health” (Van Der Oost et al. 2003). Body burdens are analytical chemical indicators, and as so they are considered to be a bioaccumulation marker (Van Der Oost et al. 2003). It is commonly accepted that, the effect of a toxic substance on an organism can be more accurately predicted based on the concentration present in the organism, rather than from the concentration at exposure site and its surrounding environment (Lock and Janssen 2001). Repeated exposure to chemicals is common in many environmental and occupational settings (Chang et al. 2005). Such long term exposures have the potential to cause bioaccumulation and raise the risk of adverse biological effects (Chang et al. 2005). This is particularly true for metals and radionuclides, since they are inorganic contaminants that are not degraded by living organisms and, therefore, can be bioaccumulated up to harmful levels (Beyersmann and Hartwig 2008). Many existing and proposed regional and international regulatory guidelines, classification schemes, and risk assessment frameworks use estimates of bioaccumulation to indicate whether substances may be hazardous in the environment (Lock and Janssen 2001). Body burdens determination is very important for the assessment of exposure to a contaminant, since it give us the information about the bioavailability of the toxicants, helping to predict potential health risks to higher trophic ecological receptors and also helping to clarify cause-effect relationships between exposure and toxic effects. However, to provide indication whether an adverse biological effect is occurring in the exposed organisms, it is essential to use also biomarkers of effect, as, for example, biomarkers of genotoxicity, immunotoxicity and tissue damage (histopathology).

1.3.1 Evaluation of biomarkers of genotoxicity

The occurrence of chemical contaminants with DNA-damaging capacity in the environment represents a threat to human health as well as to the health of ecosystems. The analysis of DNA damage in wild species has the advantage of detecting and quantifying the genotoxic contamination impact without requiring a detailed knowledge of the identity and the chemical properties of the contaminants present (Frenzilli et al. 2009).

A number of techniques have been used to identify substances with genotoxic activity, and one of the most frequently used is the comet assay. The alkaline single cell gel electrophoresis (comet) assay is one of the most sensitive methodologies used to detect DNA strand breaks. It is fast and effective, especially for small samples, it is applicable to almost all cells types, it does not require DNA extraction and purification from tissues and it allows the measurement of strand breaks in individual cells (Ostling and Johanson 1984; Singh et al. 1988; Léon et al. 2007; Frenzilli et al. 2009). This assay has proved to be an excellent and effective tool to assess the genotoxic potential of contaminants, such as metals and radionuclides in invertebrates (e.g. earthworms) (e.g. Bonnard et al. 2009; Bigorgne et al. 2010; Button et al. 2010; Lourenço et al. 2011a; Lourenço et al. 2012) and vertebrates (e.g. mice)(Scheirs et al. 2006; Léon et al. 2007).

Unrepaired DNA damage will result in the persistence of lesions in the DNA molecule that may lead to cell transformation and ultimately to cell death. These lesions may lead to chromosomal aberrations and aneuploidy (clastogenic events), which may influence the stability of the genome, further producing structural and numerical aberrations at successive cell cycle divisions (Francesca et al. 2004). These DNA abnormalities, well established as indicators of genotoxicity and citotoxicity, are currently used as biomarkers of genotoxic effects in risk assessment studies of genotoxic and carcinogenic substances (SCHER/SCCP/SCENIHR 2009). Chromosomal aberrations and aneuploidy may result in variations of cells DNA content, which can be determined by flow cytometry. Flow cytometry allows for the rapid analysis of DNA content, phenotype expression and sorting of cells for further investigations (Baatout and Derradji 2004). It allows quantitative measurements on single cells of cellular constituents at very high rates of speed (Nunez 2001; Baatout and Derradji 2004). This technique has been successfully used to evaluate the genotoxic effects of environmental pollutants to wildlife population (Bickham et al. 1998; Matson et al. 2009).

Toxicogenomics is a field, which investigates how the entire genome is involved in biological responses of organisms exposed to environmental toxicants. To that end, a number of techniques are available that can help to clarify the molecular mechanisms involved in a given process as, for

instance, DNA microarrays and Suppression Subtractive Hybridization (SSH). These techniques can help defining cellular networks of responsive genes, to identify targets of toxic response, to provide biomarkers and to identify individuals with increased susceptibility to toxicants-induced carcinogenesis and disease. SSH is based on suppression PCR and combines normalization and subtraction in a single procedure (Diatchenko et al. 1996). The normalization step equals the abundance of cDNA s within the target population and the subtraction step excludes the common sequences between the target (tester) and control (driver) populations (Diatchenko et al. 1996). This technique is particularly useful when the organisms under study do not have their genomes completely sequenced, since this previous knowledge is not necessary to perform this technique, however the same does not apply for DNA microarrays. This technique may be employed to identify down and upregulated genes after exposure to environmental contaminants. It has been successfully applied to several organisms, namely earthworms (Gong et al. 2007; Pirooznia et al. 2007), fish (Wang et al. 2007; Hagenaars et al. 2008), amphibians (Marques 2011) and microorganisms (Huang et al. 2007; De Long et al. 2008). Another very promissing technique for the determination of gene expression is quantitative real-time PCR. It allows the detection and measurement of minute amounts of nucleic acids in a wide range of samples from numerous sources and is the enabling technology par excellence of molecular diagnostics, life sciences, agriculture and medicine (Bustin et al. 2009). This technique is simple, fast, sensitive, specific and homogenous, making it a suitable test for nucleic acid quantification (Bustin et al. 2009) and for the identification and validation of genetic biomarkers (Kim et al. 2007; Kang et al. 2012).

1.3.2 Evaluation of biomarkers of immunotoxicity

The immune system is extremely vulnerable to injury by chemical pollutants. Major changes in the immune system can be expressed in considerable morbidity and even mortality of the organisms involved. Therefore, early subtle alterations in some of the components of the immune system can be used as early indicators of altered organism health (Calisi et al. 2009).The biomarkers of immunotoxicity most frequently evaluated in non-mammalian animal models, as for instance, invertebrates environmentally exposed to contaminants are: lysosomal stability, cell viability, expression of metallothioneins in hemocytes, measurement of antioxidant activity in immune cells (e.g. catalase (CAT) and superoxide dismutase (SOD)), phagocytic activity of immune cells and immune cells counts (Zelikoff 1998; Auffret et al. 2006; Fuchs et al. 2010; Fuller-Espie et

al. 2010). The measurement of the majority of these parameters is also normally done in mammals, and the main reason behind their introduction in non-mammalian systems was, the potential applicability of these organisms to serve as alternate animal models for immunotoxicological studies (Zelikoff 1998). In human studies there are other biomarkers commonly evaluated for immunotoxicological studies (Tryphonas 2001), and one of the most used is the evaluation of immune cells phenotype, designated as immunophenotyping. The evaluation of immune cell counts and immune cells phenotype is mostly done by flow cytometry (Duramad and Holland 2011). Flow cytometric immunophenotyping remains an indispensable tool to identify changes of immune cells counts and phenotype caused by the exposure to environmental contaminants, and also for the diagnosis, classification, staging and monitoring of hematologic neoplasms (Craig and Foon 2008). Flow cytometric immunophenotyping evaluates individual cells in suspension for the presence and absence of specific antigens (phenotype), using fluorescently labeled monoclonal antibodies (Craig and Foon 2008).

1.3.3 Evaluation of histopathological biomarkers

Histopathological alterations provide a sensitive indicator of sublethal stress induced by xenobiotics (Reddy and Rao 2008; Poleksic et al. 2010). Compared with reproductive and developmental changes, histological alterations are more sensitive and occur earlier (Poleksic et al. 2010), since they are considered mid-term responses (Lam and Gray 2003). Histopathological changes integrate the impact of a variety of stressors (pathogens, toxic compounds, and unfavorable nutritional and environmental conditions) (Poleksic et al. 2010), and may also signal damaging effects in organisms, resulting from prior or ongoing exposure to toxic agents (Odendaal and Reinecke 2003; Reddy and Rao 2008). Moreover, histopathological biomarkers, incorporate biotic and abiotic factors, making them reliable markers of environmental stress (Poleksic et al. 2010). Histopathological changes have been widely used as biomarkers to evaluate the health of organisms exposed to contaminants, both in the laboratory (Thophon et al. 2003) and in the field (Pereira et al. 2006; Kıllıç 2011). Several studies have used histopathological biomarkers in invertebrates, namely earthworms, to evaluate the impact of soil contaminants, namely pesticides (Muthukaruppan et al. 2005; Reddy and Rao 2008), metals (Amaral and Rodrigues 2005; Amaral et al. 2006; Giovanetti et al. 2010; Kıllıç 2011) and radionuclides (Lourenço et al. 2011b). The degree of the recorded histological changes, will always depend on the ability of

the organisms to repair the injury, the nature and severity of the contamination and the length of the exposure (Haschek and Rousseaux 1998).

1.4 Small mammals and earthworms as biological indicators of environmental pollution

Bioindicators are organisms or communities, which reactions are observed representatively to evaluate a situation, giving clues for the condition of the whole ecosystem (Gerhardt 2002). In bioindicator species, changes in presence/absence numbers, morphology, physiology or behavior will indicate that the physiological or chemical variables, to which they are exposed in the environment, are outside their preferred limits (Gerhardt 2002; Holt and Miller 2011). A broad definition of biological indicator is: "a species or a group of species that readily reflects the abiotic or biotic state of an environment represents the impact of environmental change on a habitat, community or ecosystem or is indicative of the diversity of a subset of taxa or the whole diversity within an area" (Gerhardt 2002; Holt and Miller 2011). However, mostly, bioindicators are restrictively defined as species reacting to anthropogenical effects on the environment (Gerhardt 2002; Holt and Miller 2011). According to the different applications of bioindicators there are different categories, being those focused on this thesis denominated environmental indicators. An environmental indicator is a species or group of species responding predictably to environmental disturbance or change (e.g. sentinels), aiming at diagnosing the state of the environment to support decision making processes and to be integrated in pieces of legislation aimed in defining rules for monitoring ecosystem status (Gerhardt 2002), like for example the use of macroinvertebrates as a biological group for the evaluation of water quality in the European Water Framework Directive. Earthworms and small mammals have been widely used as indicator species of environmental contamination (Pereira et al. 2006; Rogival et al. 2006; Scheirs et al. 2006; Antunes et al. 2008a; Bonnard et al. 2009; Sánchez-Chardi et al. 2009; Bernard et al. 2010).

1.4.1 *Eisenia andrei*

Earthworms are very important for the soil system, mainly because of their favorable effects on soil structure and function (Sanchez-Hernandez et al. 2006). Their burrowing and feeding activities highly contribute to increase water infiltration, soil aeration and the stabilization of soil aggregates (Sanchez-Hernandez et al. 2006). Also, these organisms help to increase soil fertility by formation of an organic matter layer in topsoil (Sanchez-Hernandez et al. 2006). During these

processes, earthworms ingest large amounts of soil, or specific fractions of soil (e.g. organic matter), which continuously expose them to contaminants present in the soil (Morgan et al. 2002; Vijver et al. 2005; Sanchez-Hernandez et al. 2006; Kilıç 2011). Due to these unique characteristics, earthworms are regarded as one of the most suitable animals for testing the toxicity of chemicals in soils (Sanchez-Hernandez et al. 2006; Lee et al. 2008) and, for many years, they have also been considered an interesting biological indicator of many metals in soils (Suthar et al. 2008). Consequently, these organisms have been adopted as standard organisms for ecotoxicological testing by the European Union (EEC 1984) and the OECD (1984). In addition, the International Standards Organization (ISO 1998, 1996) and the U.S Environmental Protection Agency (USEPA 1988) also have guidelines for toxicity tests using earthworms as test species. In several guidelines concerning these tests, *Eisenia fetida*/*andrei* were chosen because they can be easily cultured in the laboratory and because an extensive database on the effects of almost all classes of chemicals is available for these species (Lee et al. 2008). *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei Bouché* (1972) (Fig. 11) are two closely related species commonly used, for ecotoxicological testing, and for physiology and genetic studies, mainly due to their ubiquitous distribution, short life cycles, wide temperature and moisture tolerance range and resilience (Domínguez et al. 2005).

Figure 11: Photo of *Eisenia andrei* (source: Ana Luísa Caetano Photo taken in May 2012)

These species have been widely used in ecotoxicological tests for the ecotoxicological evaluation of soils contaminated with different chemicals, namely pesticides (Garcia et al. 2008; Pereira et al. 2010), nanomaterials (Canas et al. 2011; Whitfield et al. 2012), metals (Reinecke et al. 2001; Reinecke and Reinecke 2004; Homa et al. 2007; Antunes et al. 2008a; Antunes et al. 2008b; Bonnard et al. 2009; Giovanetti et al. 2010) and radionuclides (Lourenço et al. 2011a; Lourenço et al. 2011b; Lourenço et al. 2012) in a single form or as part of complex mixtures. Nevertheless, few studies evaluate the effects of radionuclides.

1.4.2 *Apodemus sylvaticus*

Free living wild animals are often used to monitor the bioavailable fraction of contaminants and their potentially hazardous consequences (Beernaert et al. 2007). Wild populations inhabiting polluted sites are often exposed to a mixture of chemical pollutants which are mainly uptaken with food or water and/or air inhaled (Sánchez-Chardi et al. 2009). Among these organisms, small mammals were preferentially selected, as they accumulate pollutants that are present in the environment and also because they sensitively respond to various changes in terrestrial ecosystems (Festa et al. 2003; Mukhacheva et al. 2010). Moreover, small mammals such as rodents are used to monitor exposure risks, because they allow a better assessment of the effects on consumers of high biological complexity and therefore allow the prediction of possible risks to human health (Marcheselli et al. 2010). For small mammals to be used as indicators of pollution, they should fulfill specific criteria such as: high abundance, low rate of migration and a reasonable life expectancy (Marcheselli et al. 2010). The wood mouse (*Apodemus sylvaticus*) (Fig. 12) fulfills the above mentioned criteria (Rogival et al. 2006; Sánchez-Chardi et al. 2009; Marcheselli et al. 2010). It is a widespread species, abundantly present in the nature, mainly a primary consumer, although occasionally insectivorous (Sánchez-Chardi et al. 2009; Martiniaková et al. 2010). The *Apodemus sylvaticus* species is frequently used for pollution monitoring, mainly for metal pollution (Festa et al. 2003; Pereira et al. 2006; Rogival et al. 2006; Beernaert et al. 2007; Sánchez-Chardi et al. 2009; Marcheselli et al. 2010; Martiniaková et al. 2010; Mukhacheva et al. 2010)

Figure 12: *Apodemus sylvaticus* (source: reddeparquesnacionales.mma.es)

The use of wild specimens in ecotoxicological studies includes aspects of bioavailability, toxicity, detoxification mechanisms, specific or individual exposure and susceptibility as determining factors for environmental risk assessment under natural conditions (Sánchez-Chardi et al., 2009). Biomonitoring pollution through wild animals is crucial for the assessment of environmental quality and to improve our understanding of the response capacity of natural populations to pollution (Sánchez-Chardi et al., 2007). Also data collected from vertebrates living in contaminated areas, are very important to infer about potential human and environmental risks, since they provide information on potential wildlife and human hazard effects (Pereira et al., 2006).

1.5 Biomonitoring of populations living nearby uranium mines

Monitoring of pollutants, their derivatives and related substances, which may induce chemical, physical or biological stress, is a key issue of public health (Schmidt et al. 2011). Populations inhabiting nearby tailings and mining activities may be exposed to a variety of hazardous materials, which may increase the risks for health problems (Au et al. 1995). Exposure to environmental uranium has been linked with several heath consequences, including cancer, nephrotoxicity and respiratory diseases (Wagner et al. 2010). Its presence in the environment as a byproduct of various anthropogenic activities has the potential to exert toxic effects on several

important physiological processes, including kidney function, bone development and hematopoiesis (Brugge et al. 2005; Wagner et al. 2010; Brugge and Buchner 2011). Uranium accumulation in the bone directly affects its structure and metabolism (Wagner et al. 2010). The bone marrow is also affected by uranium, and when this element is sequestered in the bone it has the potential to alter hematopoiesis (Wagner et al. 2010). Uranium's nephrotoxicity can compromise kidney function by disturbing renal proximal tubule reabsorption, therefore, it may not only affect hematological parameters directly through irradiation of bone marrow, but also indirectly by acting on the kidneys and potentially reducing erythropoietin production (Wagner et al. 2010). Most of the studies performed to date, have assessed the health risks associated with working in uranium mines, mills, or processing facilities (Schneider et al. 1999; Popp et al. 2000; Milacic and Simic 2009), few have examined non-occupationally exposed groups, like residents from areas where environmental releases of uranium or other metals and radionuclides have occurred (Wagner et al. 2010; Brugge and Buchner 2011).

In Portugal there are several abandoned uranium mines, located mainly in the north and center regions of the country, and a great fraction of them, are located near human inhabited regions. According to Portuguese legislation (Decreto Lei nº 198A/2001, 6 de Julho; Resolução da Assembleia da República n.º 34/2001), mining companies which have received authorization for ore extraction, have to guarantee safe and secure discharges and deposition of waste materials in the environment, as well as the environmental remediation of sites after the cessation of mining operations (Carvalho et al. 2005a). However, and regarding uranium mines specifically, when the uranium exploitation ceased in the country, the company entered in liquidation (Carvalho et al. 2005a). Therefore, the proper closure and remediation of the majority the uranium mines existing in the country, was not performed, leaving tonnes of tailings, heaps, and acid/radioactive effluents without any treatment. As a consequence, for years, populations living nearby abandoned uranium mines, were exposed to radioactive wastes. At some point, the understanding of the risks to human health lead the government to consider the recovery of mining areas as a matter of public interest, and also to establish a list of priority areas (Despacho conjunto nº 242/2002; Despacho conjunto nº 267/2005) to be subjected to remediation works. As a result, a public company was created (EDM – Empresa de Desenvolvimento Mineiro, SA), to manage these mines and, in due time, proceed with the remediation process. To date, the only study performed in Portugal, with the purpose of determining the risk of population's exposure to these hazardous wastes, was the MinURAR project, performed in 2005 in the population of Urgeiriça (Falcão et al. 2005). Urgeiriça mine is the biggest one in the country, but it is not the

only one that represents serious risks to human health and surrounding natural resources and ecosystems. As so, more studies are required, in other abandoned mines dispersed throughout the country, to help the evaluation of the real scenario and provide a more realistic picture of the potential hazardous effects of radioactive wastes in Portuguese populations.

1.6 Scope and objectives of this thesis

The Cunha Baixa uranium mine has been under study for quite some time, by our team. The studies mainly aimed to characterize soil, sediments and water from the mine pit, the water treatment settling pond, wells and public water supplies in terms of their radiological and chemical properties (Carvalho et al. 2005a; Carvalho et al. 2005b; Neves et al. 2005; Pereira et al. 2008; Carvalho et al. 2009a); ecotoxicological evaluations with invertebrates, algae and plants were performed to soils surrounding the mine, to wastewaters from its associated ponds, including the effluent treatment pond (Antunes et al. 2007a; Antunes et al. 2007b; Antunes et al. 2008a; Antunes et al. 2008b); the phytotoxicity and genotoxicity of the soils surrounding the mine were tested using important and sensitive crop species and also using the Ames test for soils elutriates (Pereira et al. 2009); the genotoxicity, histopathological effects, antioxidant responses and metal bioaccumulation were also assessed in Iberian gree frogs (*Pelophylax perezi*) inhabiting the main acidic pond and the contaminated sludges removed from the effluent treatment pond (Marques et al. 2008; Marques et al. 2009).

This thesis is part of this comprehensive study performed in the Cunha Baixa uranium mine. Studies were conducted to determine, whether the residues from the Cunha Baixa uranium mine represent a chemical and radiological risk to indicator species like earthworms and small mammals, but also to the population living nearby, by inducing biological effects at the molecular and cellular level, that could lead to the development of serious chronic diseases. It was also aimed to determine if earthworms and wood mice could be good indicator species for the detection of genotoxic effects caused by the exposure to the contaminated residues from the Cunha Baixa uranium mine. Additionally, it was also aimed to detect and clarify underlying mechanisms triggering responses to metals and radionuclides exposure, by pointing out for the development of potentially new molecular biomarkers. By studying the human population living nearby the abandoned mine it was intended to identify early warning signals of molecular and cellular damages, which could increase the risk of development of genetic diseases, such as cancer and also potentially affect the genetic integrity of germ cells leading to decreased fertility

and increased incidence of childhood genetic diseases and birth defects. Finally, and in face of the results obtained, alert the authorities for the real problem of radioactive wastes and abandoned uranium mines.

To answers the questions raised at the beginning of this thesis, several experiments were conducted. In each chapter, the specific objectives of each experiment, the methodologies employed, the results obtained and their critical discussion are described.

Chapter 2: With the purpose to investigate the genotoxic and cytotoxic effects of metals and radionuclides in earthworms, organisms were exposed to sludge from the water treatment settling pond, heavily contaminated with metals and radionuclides and to the reference soil LUFA 2.2. Parameters like DNA damages in coelomocytes, coelomcytes frequency, growth and bioaccumulation of metals and radionuclides were evaluated. Results showed genotoxic and cytotoxic effects in earthworm's coelomocytes, growth inhibition and also bioaccumulation of metals and radionuclides by earthworms exposed to the contaminated soil.

Chapter 3: In order to determine histopathological responses to the exposure to metals and radionuclides, earthworms were exposed in the laboratory to sludge from the waste water treatment pond and to LUFA 2.2. Earthworm's transversal cuts were stained using the hematoxilin-eosin method. Results showed severe alterations in the epidermis, muscle tissue, chloragogenous tissue and intestinal epithelium in earthworms exposed to the contaminated soil.

Chapter 4: Aiming to evaluate genotoxic and cytotoxic effects of *in situ* exposure to uranium mining wastes, earthworms were exposed to sludge from the water treatment settling pond and to the reference soil LUFA 2.2, in containers placed in the sludge deposition area near the mine pit and in a reference area. DNA damages in coelomocytes, coelomocytes frequency, median fluorescence intensity, proliferation and DNA content were determined, as well as growth, reproduction and bioaccumulation of metals and radionuclides. Result showed genotoxic and cytotoxic effects in earthworm's coelomocytes, growth and reproduction inhibition and also metals and radionuclides bioaccumulation.

Chapter 5: In order to identify the main biological pathways and cellular physiological functions affected by the exposure to soil contaminated with metals and radionuclides, as well as potentially new genetic biomarkers, the gene expression profile of earthworms exposed *in situ* to

a contaminated and a reference soil, was determined. Results showed that the main physiological functions affected were metabolism, oxireductase activity, redox homeostasis and response to chemical stimulus and stress. Results also showed that *NADH dehydrogenase subunit 1* and *elongation factor 1 alpha* were significantly altered in response to metals and radionuclides. In this work it was also identified a sequence with homology for oncogene *SET*, described for the first time in earthworms.

Chapter 6: With the purpose to evaluate the genotoxic effects occurring in the European wood mouse inhabiting the deposition area of uranium mining wastes, mice were captured in the contaminated and reference areas. DNA damages, gene expression profile and single nucleotide polymorphisms in tumour suppressor genes and bioaccumulation of metals in target organs were determined. Results showed DNA damages, alteration of the expression of *P53* gene, single nucleotide polymorphisms in the *Rb* gene and bioaccumulation of uranium, cadmium and copper.

Chapter 7: Aiming to determine the risk of uranium mining wastes exposure of the population inhabiting in the village of Cunha Baixa nearby the Cunha Baixa uranium mine, blood samples were collected and analysed to evaluate genotoxicity, immunotoxicity and the presence of trace elements. Results showed DNA damages, depletion of natural killer cells and T lymphocytes and also increased levels of uranium and manganese and decreased levels of zinc in blood samples.

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CHAPTER 2 - GENOTOXIC ENDPOINTS IN THE EARTHWORMS SUB-LETHAL ASSAY TO EVALUATE NATURAL SOILS CONTAMINATED BY METALS AND RADIONUCLIDES

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2.1 Abstract

Eisenia andrei was exposed, for 56 days, to a contaminated soil from an abandoned uranium mine and to the natural reference soil LUFA 2.2. The organisms were sampled after 0, 1, 2, 7, 14 and 56 days of exposure, to assess metals bioaccumulation, coelomocytes DNA integrity and cytotoxicity. Radionuclides, bioaccumulation and growth were also determined at 0 h, 14 and 56 days of exposure. Results have shown the bioaccumulation of metals and radionuclides, as well as, growth reduction, DNA damages and cytotoxicity in earthworms exposed to contaminated soil. The usefulness of the comet assay and flow cytometry, to evaluate the toxicity of contaminants such as metals and radionuclides in earthworms are herein reported. We also demonstrated that DNA strand breakage and immune cells frequency are important endpoints to be employed in the earthworm reproduction assay, for the evaluation of soil geno and cytotoxicity, as part of the risk assessment of contaminated areas. This is the first study that integrates DNA damage and cytotoxicity evaluation, growth and bioaccumulation of metals and radionuclides in a sub lethal assay, for earthworms exposed to soil contaminated with metals and radionuclides.

Keywords: *Eisenia andrei*; DNA damages; metals; radionuclides; cytotoxicity

2.2 Introduction

In the last few years, attention has been focused on impacts on human health and on the natural environment promoted by uranium mining and all the nuclear fuel cycle (IAEA, 2005). The risks of uranium mill tailings are associated with their content in metals and radionuclides, raising concern among the general public and governmental authorities — in some places exacerbated by official secrecy and lack of data on health impacts (IAEA, 2005). Human and other living species from uranium mining districts may be exposed: i) to radiation doses emitted by wastes; ii) radioactive dust; iii) contaminated water and foodstuffs (IAEA, 2005). The determination of the risks of such hazards is usually carried out through chemical analysis of environmental samples, neglecting the assessment of its biological effects. Regarding soil analysis, this fact seems to be even more important, since chemical analysis cannot consider issues like mixture toxicity and the role of soil properties (such as soil structure and organic matter, pH, etc.) in determining chemical's bioavailability (Gastaldi et al., 2007; Lee et al., 2009). These are determining factors for their toxicity to soil organisms (Gastaldi et al., 2007; Bonnard et al., 2010). The best integrators of these complex effects are exposed organisms themselves (Gastaldi et al., 2007; Lee et al., 2009).

Among soil organisms, earthworms are of particular interest, since they are powerful regulators of soil processes, participating in the maintenance of its structure and regulation of organic matter dynamics (Lavelle et al., 1997). Moreover, these organisms are important agents in modulating the transfer of inorganic and organic toxicants throughout the food chains, due to their presence in contaminated soils (Cooke et al., 1992). For many years they have been considered an interesting biological indicator of the presence and bioavailability of many metals in soils (Suthar et al., 2008). In general, earthworms are effective accumulators of metals, followed by compartmentalization, storage, or excretion of metal ions from the most sensitive tissues (Lukkari et al., 2004). The importance of earthworms to test the potential adverse effects of chemicals on soil organisms has been recognized by various environmental organizations and resulted in the publication of standard test guidelines (OECD, 1984, 2004; ISO, 2005) aimed in evaluating soil habitat function. Standard toxicity tests evaluate parameters such as mortality, growth and reproduction, which may lead to the underestimation of risks posed by the exposure to compounds with both, chemical and radiological activity. Since effects at a molecular and cellular level may also occur, without being immediately translated in effects at the individual level, molecular biomarkers can be a complementary approach providing information about organism's stress response to toxicants and mixtures (Bonnard et al., 2009). The use of biomarkers is becoming increasingly important in the evaluation of the effects of contaminants in

earthworms, promoting the discussion on the need to develop new biomarkers for these organisms, to supplement the evaluation of responses in standard toxicity tests (Reinecke and Reinecke, 2004; Gastaldi et al., 2007). Regarding uranium radiotoxic and chemotoxic properties, new endpoints to evaluate cellular and DNA damages seem to be highly relevant (Svendsen et al., 2004; Barillet et al., 2005), since uranium acts on the formation of oxidative DNA damages.

Since DNA is an important target of environmental stress (Frenzilli et al., 2001) the possibility of adding biomarkers of genotoxicity and cytotoxicity, to the list of endpoints presently used to assess soil habitat function, was investigated. More specifically, flow cytometry and comet assay were employed to detect alterations on cell populations' frequency and DNA integrity in coelomocytes, caused by the exposure to metals and radionuclides in earthworms. Two main cohorts or lineages of coelomocytes, namely amoebocytes and eleocytes, are present in various proportions in different earthworm species (Kurek and Plytycz, 2003). Amoebocytes derive from the mesenchymal lining of the coelom (Plytycz et al., 2006), while eleocytes (chloragocytes) differentiate from the chloragogen cells that cover the coelomic surfaces of the alimentary tract and major blood vessels (Affar et al., 1998; Peeters-Joris, 2000).The study of coelomocytes in earthworms is relevant since these are immunocompetent cells suspended and circulating in the coelomic fluid constituting the hydrostatic skeleton of oligochaete annelids, including earthworms (Plytycz et al., 2006). These leukocytes that surround the intestinal tract are particularly exposed to soil pollutants and are involved in the process of cell immunity (Dhainaut and Scaps, 2001; Manerikar et al., 2008).

In parallel with the evaluation of cellular biomarkers and, to clarify cause-effect relationships, the bioaccumulation of metals and radionuclides by earthworms was assessed.

2.3 Materials and Methods

2.3.1 Soils tested

Two soils were selected for the study: the standard natural soil, LUFA 2.2 as control/reference soil (Speyer, Germany), and a contaminated soil from the Cunha Baixa uranium mine (Centre of Portugal). In this mine area, soil contamination results from the deposition of mine tailings and sludge from the effluent treatment pond and from runoffs from the aquatic system (Antunes et al., 2008b). The soils from the Cunha Baixa uranium mine area, have been well characterized by Pereira et al. (2008). Based in this previous study, the soil from site B was selected for these experiments. This soil has received sludge from the treatment pond which is highly contaminated

with metals and radionuclides, because it is formed by the precipitation of radionuclides from the mine effluent with barium chloride (Pereira et al., 2008). Thus this sludge has also high extractable concentrations of metals (namely U, Mn, Al and Sr) (Pereira et al., 2008) and is toxic for *E. andrei* (Niemayer et al. personal communication).

After discarding the superficial layer (plant debris), the first 20 cm of soil were collected and sieved to discard the >2 mm fraction. Prior to the test, pH, water content (%) and water holding capacity (WHC) of both soils were measured and soil water content was adjusted to 40% of WHC_{max}. Soil pH was measured in a soil-water suspension (1:5 w/v extraction ratio) according to a described method (FAOUN, 1984). Soil water content was determined from weight loss after drying the soils at 105 °C, for 24 h. Water holding capacity (WHC) of soils was determined as described in ISO (2005).

2.3.2 Test organism

The earthworms (*Eisenia andrei*) used for the assay were obtained from a synchronised laboratorial culture, reared in large containers with a substrate composed by peat moss, horse manure and $CaCo_3$ (to adjust the pH between 6 and 7), under temperature 20±2 °C and photoperiod 16 h^L: 8 h^D). The culture medium is periodically moistened and the pH monitored. The organisms are fed twice a month with dry and defaunated manure. According to international standard guidelines (OECD, 2004; ISO, 11268-2:1998), adult earthworms with clitellum and body mass between 250 and 600 mg were used in this study. Before exposure, the organisms were acclimated for 24 h in containers with LUFA 2.2.

2.3.3 Laboratory exposure

Organisms were exposed to soils in 1-L plastic buckets (12 cm height and 10 cm in diameter), with lids bearing one opening at the top (Antunes et al., 2008a). The openings were covered with 300 mm nylon mesh, using white thermal glue (supplied by Elis–Taiwan, Taiwan, ref. TN122/WS), which has been shown to be non-toxic to aquatic invertebrates (Pereira et al., 2000).

For the experiment, 37 chambers were used for each soil tested (a total of 74 chambers), each containing 10 organisms and 500g of dry weight equivalent of soil. The earthworms of 25 of these 37 containers were used for the comet assay, flow cytometry and metal body burdens analysis. The organisms from the remaining 12 containers were analyzed for radionuclides

bioaccumulation. For all the analyses performed, animals were sampled before exposure (0 days), directly from acclimation containers with LUFA 2.2 soil. For comet assay, flow cytometry and metal body burdens, 5 test chambers were sampled for each exposure period: 1, 2, 7, 14 and 56 days. Following the protocol of Maenpaa et al. (2002), the animals used for metal body burdens assessment were left to depurate for ±6 h in moistened filter paper and kept at -20ºC until analysis. For radionuclide's content analyses, 6 test chambers were sampled for each exposure period: 14 and 56 days. To allow total clearance of the gut content, the animals were left to depurate for 48 h in moist filter paper. Afterwards they were weighted (fresh weight) and stored at -80ºC until analysis. Organisms were weight to the nearest 0.1 mg (fresh weight), and radionuclide's contents as well as the growth of organisms was determined.

During the experiment, organisms were fed, once a week, with 5 g *per* test chamber of horse manure. The experiment was carried out under a controlled environment (temperature 20±2 °C; photoperiod 16^{L}: 8^{D}). After 28 days of exposure, the test soils were renewed in all test chambers, and only adult earthworms were moved to the new soil. Such procedure aimed to prevent the hatching of cocoons produced, during this period, to avoid competition between juveniles and adults.

2.3.4 Radionuclides and metal body burdens

For radionuclide's analysis, earthworms were prepared according to the methodology described by Carvalho et al. (2007; 2009).

Quantification of metal body burdens was performed with slight modifications of the methodology described by Antunes et al. (2008a). A pool of 4 earthworms for each replica (5 replicas per exposure period and soil tested) was used and the digest was diluted to a final volume of 5 ml with Mili-Q water. Sample blanks were obtained following the same procedure, but without the biological sample. Inductively coupled plasma mass spectrometry (ICP/MS) (APHA-AWWA-WEF, 1995) was employed to determine the total concentrations of Be, Al, Ba, Mn, Fe, Ni, Zn, Sr, Se, Cd, Pb and U in whole body of earthworms exposed to LUFA 2.2 and to the contaminated soil. Results were expressed in $\mu g\ g^{-1}_{dw.}$

To determine metal bioaccumulation factor (BAF) for earthworms, the ratio between the metal body burden in earthworms ($\mu g\ g^{-1}$ dry weight) and total concentration in soil ($\mu g\ g^{-1}$ soil) was calculated for each metal, in all the exposure periods.

For calculation purposes, the total metal concentrations found in soil B, by André and co-authors (2009) reporting a study carried out in parallel, were used.

2.3.5 Coelomocytes extrusion

Earthworm coelomocytes were obtained using the modified protocol of Reinecke et al. (2004). The same cell suspensions were used either for comet assay and flow cytometry analysis. Before use, the cell suspension was divided in two aliquots which were frozen at -80ºC in a cryopreservation medium, previously tested, containing 10% dimethyl sulfoxide (DMSO) + phosphate buffered saline (PBS), and 10% Glycerol + PBS + 10% fetal bovine serum (FBS), for comet assay and flow cytometry analysis, respectively

2.3.6 Comet assay

Prior to comet assay, the cell suspension was placed at 37ºC to rapidly defrost, centrifuged 3 min at 380*g* and washed with PBS. The comet assay was conducted under yellow light, to prevent UV-induced DNA damage, and performed with slight modifications of the protocol described by Nogueira et al. (2006): briefly, microscope slides, were covered with the first agarose layer and left to dry; another layer containing the cells was placed on top of the first agarose layer.

Visual scoring of cellular DNA on each slide was based on the categorization of 100 randomly-selected cells. The comet-like formations were visually graded into five classes, depending on DNA damage and scored as described by García et al. (2004).

Positive controls were always included, and consisted of cells, previously exposed to 200 μM of H_2O_2, for 1 h.

2.3.7 Flow cytometry

Prior to flow cytometry analysis, cells were placed at 37ºC to rapidly defrost, centrifuged 3 min at 380*g* and washed with PBS. Coelomocytes with different characteristics were separated and analysed through cell sorting using a FACSAria™ (BD Biosciences, Erembodegem, Belgium). After sorting, cells groups were analysed and characterized through light microscopy.

After the identification and characterization of coelomocytes, the analyses of samples, were performed on a FACSCanto™ II (BD Biosciences, Erembodegem, Belgium). During analytical

experiments, 100,000 threshold events per worm sample were collected and analyzed on the basis of their size and complexity. The resulting files were analysed using Infinicyt 1.2 software (Cytognos).

2.3.8 Statistical analysis

The effect of exposure time and soil on the parameters analysed was tested trough a bifactorial ANOVAs, followed by Dunnett multi-comparison tests (when applicable) to discriminate significant differences between organisms exposed to a given period and the control group [organisms before exposure (0h)]. Whenever a significant interaction between both factors was recorded Student's t-tests were also used to compare organisms exposed to both soils for each exposure period. The level of significance defined for all the analyses was 0.05.

2.4 Results & Discussion

Biological systems are the target for the action of toxicants, and thus provide important information which cannot be obtained from chemical analyses of the environmental samples. Thus, they could be used as diagnostic tools for integrated environmental evaluation (Jha, 2008). This highlights the importance of assessing biological responses as an integral part of hazard and risk assessment (Moore et al., 2004).

Herein, the contaminated soil (soil B), was characterized by a pH of 7.79 and 7.71 ± 0.60 % (Table 1) of organic matter (see Pereira et al., 2008). These characteristics are not favourable to the bioavailability of metals (Lukkari et al., 2004; Antunes et al., 2008a; Pereira et al., 2008) in the soil solution. However, almost all the metals analysed (Be, Al, Ba, Mn, Fe, Ni, Zn, U) were found in significant higher concentrations (Table A1, please see Supplementary Material section) in the earthworms exposed to the contaminated soil (Table 2). As suggested by (Hobbelen et al., 2006), this may result from the direct exposure of the organisms to the metals through dermal contact in the soil solution or by ingestion of water, polluted food and/or soil particles. Since pH is variable in the different compartments of their gastrointestinal tract, it may increase the mobilization of contaminants from soil (Peijnenburg and Jager, 2003; Li et al., 2009). Despite all these possibilities, data presented by Peijnenburg et al. (1999) suggest a lower impact of soil characteristics on metal uptake in *E. andrei* compared to other soil dwelling invertebrates, namely *Enchytraeus crypticus*. The levels of most of the metals analyzed started to decrease after 14 days of exposure (Table 2).

Table 1: Main physico-chemical properties and pollutant concentrations in both the contaminated soil (soil B) and the reference LUFA 2.2 soil

	Lufa 2.2	**Soil B**
pH	5.89 ± 0.26	7.79 ± 0.01
Conductivity (μS/cm)	49.17 ± 2.94	2263 ± 11.55
Moisture (%)	2.28 ± 0.81	48.2 ± 0.12
Organic matter (%)	3.61 ± 0.33	7.71 ± 0.60
Metals (μg/g soil)		
Al	3656.33 ± 196.09	26440 ± 1109.77
Ba	48.21 ± 6.93	8.50 ± 14.04
Be	0.34 ± 0.03	50.11 ± 4.29
Cd	0.09 ± 0.01	2.58 ± 0.23
Fe	3345 ± 635.24	13383.33 ± 654.44
Mn	119.38 ± 7.83	3711.33 ± 103.27
Ni	2.62 ± 0.39	91.38 ± 1.48
Pb	10.26 ± 0.32	9.72 ± 0.71
Sr	8.68 ± 0.12	19.29 ± 12.41
U	1.08 ± 0.49	215.72 ± 8.50
Zn	13.01 ± 0.52	511.73 ± 4.94

(for further details please see Pereira et al. 2008 and André et al. 2009)

The metals which levels continuously increased throughout the exposure period are essential elements, like Zn or Se, or non-essential elements, difficult to eliminate, like Cd or Pb (Table 2). Standard toxicity protocols were not designed to assess body burden and consequently there are no internationally agreed standards for depuration time (Nahmani et al., 2007). Commonly earthworms are depurated onto filter paper for periods of 24 or 48 h but it ranges from 6 to 96 h (Arnold and Hodson, 2007; Nahmani et al., 2007). The data collected from preliminary earthworm gut clearance experiments conducted by Maenpaa et al. (2002) at 6, 12, or 24 h showed that the main content of the gut is cleared in 6h. Furthermore, this was confirmed by the histopathological analysis of earthworms tissues performed by our team (data not shown), that showed total gut clearance after 6h of depuration, since soil residues were not detected in the gastrointestinal tract of these organisms. As suggested by Maenpaa et al. (2002) longer gut clearance time may result in depuration of metals from earthworm tissues and little additional clearance of soil from the earthworms.

BAFs values showed that barium was the element with highest BAF in the organisms exposed to the contaminated soil (Table 3), which may be explained by the fact that this soil was collected at a sludge deposition site. The sludge is originated from a treatment pond, where contaminants of the mine effluent are neutralized and radionuclides are precipitated, through chemical

treatment with lime and barium chloride. However, these considerations may not be straightforward, since this soil display a complex mixture of contaminants, whose interactive effects are unknown (Antunes et al., 2008a).

Environmental exposure to sources of radiation has raised great concern and thus more information on the transfer and accumulation of radionuclides in the biological compartments of ecosystems is required (Yoshida et al., 2005). The International Commission on Radiological Protection (ICRP) is planning to select earthworm as one of the reference organisms to be used in their radiation protection recommendations (Yoshida et al., 2005). The elemental composition of earthworms gives useful information on background levels and possible accumulation of toxic metals as well as related radionuclides (Yoshida et al., 2005). Although accumulation of radiocesium was studied by several scientists as summarized by Brown and Bell (1995), the accumulations of other radionuclides have been reported in only a limited number of references. To the best of our knowledge this is the first study reporting the bioaccumulation of the naturally occurring radionuclides, from the uranium and thorium decay series, ^{238}U, ^{235}U, ^{234}U, ^{230}Th, ^{226}Ra, ^{210}Pb, ^{210}Po and ^{232}Th in earthworms exposed to mine tailings from an abandoned uranium mine. These radionuclides are among the most important alpha and beta emitters (Jia et al., 2009) and they are naturally distributed (Rodríguez et al., 2010). The exploitation of materials that contain these natural radionuclides, such as, for instance, uranium mines, may lead to an increase in their concentration levels or to enhanced exposure (Rodríguez et al., 2010). The soil used in the present study is contaminated by sludge from the neutralization pond of acid waters from the underground mine of Cunha Baixa (Carvalho et al., 2007). It was reported by Carvalho et al. (2007) that the sludge resulting from the mine effluent treatment contains high concentrations of radionuclides from uranium series, low concentration of radionuclides from thorium series and high radioactivity levels. When exposed to the soil contaminated with sludge, earthworms accumulated all the radionuclides analyzed (except for ^{232}Th), however, some differences were noticed among the accumulation profiles (Table 4). The composition of this soil (described elsewhere) (Carvalho et al., 2007), may also explain the low levels of ^{232}Th in the earthworms analyzed. The accumulation of the radionuclides ^{238}U, ^{234}U, ^{235}U and ^{210}Po, increased throughout the exposure period. This may be related to the fact that, after entering the soil, radionuclides often become associated with organic matter and fine particles, which form the main food source for detritivorous invertebrates (Wood et al., 2009).

Table 2: Metal contents (µg g-1dry weight) (mean ± standard deviation, n = 5) in *Eisenia andrei*. In the same column, letter (a) stand for statistical significant differences after a Dunnet's multi-comparis test (p<0 05, following two-way ANOVA) and letter (b) for statistical significant differences between soils after a t-test, for each exposure period (p<0.05).

Soils	Time	Be	Al	Mn	Fe	Ni	Zn	Se	Sr	Cd	Ba	Pb	U
Earthworm metal content ± SD (µg.g^{-1} dry weight)													
Before exposure (0 d)		0.12 ± 0.02	1544 ± 369.9	87.6 ± 18.5	1793 ± 368.4	1.6 ± 0.3	103 ± 12.6	2.4 ± 0.6	13 ± 1.2	3.4 ± 2.6	24 ± 4.1	5.9 ± 1.4	0.38 ± 0.03
Lufa 2.2	1 d	0.13 ± 0.05	1172 ± 378.3	96.9 ± 19.5	1392 ± 428.7	1.2 ± 0.3	114 ± 10.15	2.5 ± 0.6	11 ± 0.9	1.8 ± 0.7	19 ± 4.8	5.3 ± 2.4 $^{(b)}$	0.35 ± 0.06
	2 d	0.14 ± 0.04	1323 ± 521	88 ± 30.9	1476 ± 705.2	1.3 ± 0.4	117 ± 19.4	2.7 ± 0.3	12 ± 2.4	1.7 ± 0.3	23 ± 7.7	6.3 ± 2.3$^{(b)}$	0.5 ± 0.14
	7 d	0.13 ± 0.03	1237 ± 394.7	81 ± 22.5	1372 ± 442.2	1.1 ± 0.4	120 ± 11.6	2.5 ± 0.4	13 ± 1.2	1.7 ± 0.2	21 ± 4.8	8.2 ± 4.2$^{(b)}$	0.4 ± 0.08
	14 d	0.11 ± 0.02	1219 ± 201.1	76 ± 9.5	1381 ± 283.4	1 ± 0.3	106 ± 11.5	3.3 ± 2.8$^{(a)}$	11 ± 0.9$^{(a)}$	2.1 ± 0.4	21 ± 2.3	7 ± 2.9	0.6 ± 0.2
	56 d	0.15 ± 0.01	1262 ± 188.5	78 ± 12.3	1373 ± 195.6	1.2 ± 0.2	103 ± 11.2	5.5 ± 0.5$^{(a)}$	12 ± 0.6	3.6 ± 0.7	20 ± 2.4	6.1 ± 0.9	0.5 ± 0.06
Soil B	1 d	9.16 ± 1.9$^{(a,b)}$	5026 ± 1091.3$^{(a,b)}$	796 ± 160.5$^{(a,b)}$	3575 ± 732.5$^{(a)}$	17.6 ± 3.6$^{(a,b)}$	222 ± 32.9$^{(a,b)}$	3.5 ± 0.9	16 ± 2.5$^{(b)}$	3.4 ± 1.5	130 ± 33$^{(b)}$	2.4 ± 0.5$^{(a)}$	59 ± 11.3$^{(a,b)}$
	2 d	6.35 ± 4.02 $^{(a,b)}$	3467 ± 2351 $^{(a)}$	529 ± 18.5$^{(a,b)}$	2383 ± 1537.9	12 ± 7.4$^{(a,b)}$	213 ± 36.7$^{(a,b)}$	2.8 ± 0.5	13 ± 2.6	2 ± 0.5	118 ± 72.5$^{(b)}$	1.9 ± 0.9 $^{(a)}$	47 ± 28$^{(a,b)}$
	7 d	11.25 ± 1.54$^{(a,b)}$	6252 ± 740.2 $^{(a,b)}$	1040 ± 115.9$^{(a,b)}$	4196 ± 586.1$^{(a,b)}$	22 ±2.9$^{(a,b)}$	263 ± 15.4$^{(a,b)}$	2.8 ± 0.3	14 ± 0.6	1.9 ± 0.1	148 ± 16.9$^{(b)}$	2.9 ± 0.5$^{(a)}$	91 ± 13.8$^{(a,b)}$
	14 d	9.45 ± 3.03$^{(a,b)}$	5064 ± 1660.3$^{(a,b)}$	930 ± 314.1$^{(a,b)}$	3471 ± 1129.3$^{(a)}$	19 ± 6.1$^{(a,b)}$	252 ± 28.2$^{(a,b)}$	2.8 ± 0.6	13 ± 1.3$^{(b)}$	1.9 ± 0.3	130 ± 47.8$^{(b)}$	3.9 ± 2	77 ± 23.1$^{(a,b)}$
	56 d	7.09 ± 4.15$^{(a,b)}$	3460 ± 2016.9$^{(a,b)}$	697 ± 431.4$^{(a,b)}$	2770 ± 1299.3$^{(a)}$	13 ± 8.9$^{(a,b)}$	292 ± 118.4$^{(a,b)}$	8.8±1.8$^{(a,b)}$	13 ± 6	3.9 ± 1	101 ± 54.9$^{(b)}$	4 ± 2.7	74± 35.7$^{(a,b)}$

Table 3: Bioacumulation factors (BAF) in *Eisenia andrei*, after 1, 2, 7, 14 and 56 days of exposure to the contaminated soil from the Cunha Baixa uranium mine (soil B).

Time	Be	Al	Mn	Fe	Ni	Zn	Sr	Cd	Ba	Pb	U
1 day	0.18	0.19	0.21	0.27	0.19	0.43	0.8	1.32(*)	15.24(**)	0.25	0.27
2 days	0.13	0.13	0.14	0.18	0.13	0.42	0.67	0.78	13.86(**)	0.20	0.22
7 days	0.22	0.24	0.28	0.31	0.24	0.51	0.74	0.76	17.36(**)	0.30	0.42
14 days	0.19	0.19	0.25	0.26	0.21	0.49	0.67	0.75	15.24(**)	0.41	0.35
56 days	0.14	0.13	0.19	0.21	0.15	0.57	0.66	1.5(*)	11.84(**)	0.41	0.34

Table 4: Radionuclide concentrations (Bq kg^{-1} dry weight) (mean ± standard deviation) in *Eisenia andrei*, before exposure (0), after 14 days of exposure (14) and after 56 days of exposure (56).

Soils	Time	^{238}U	^{235}U	^{234}U	^{230}Th	^{226}Ra	^{210}Pb	^{210}Po	^{232}Th
Worm radionuclides concentration ± SD (Bq kg^{-1} dry weight)									
Before exposure (0d)		2.5 ± 0.2	0.14 ± 0.04	3.4 ± 0.2	0.24 ± 0.04	2.4 ± 0.3	6±0.5	26.6±1.5	0.24 ± 0.05
Lufa 2.2	14 d	3.5 ± 0.2	0.14 ± 0.04	4.5 ± 0.2	1.0 ± 0.2	2.9 ± 0.4	3.5±0.4	23.6±1.4	0.56 ± 0.14
	56 d	4.8 ± 0.3	0.22 ± 0.05	6.2 ± 0.3	3.8 ± 0.3	2.5 ± 0.3	4.3±0.4	22.8±1.5	0.78 ± 0.11
Soil B	14 d	110 ± 3	4.7 ± 0.4	110 ± 3	99 ± 5	29.8 ± 1.2	25.7±2.5	108.2±3.3	0.23 ± 0.06
	56 d	145 ± 6	6.6 ± 0.8	153 ± 6	30.2 ± 1.5	19.7 ± 1.4	13.6±1.0	128±3.8	<0.20

As for ^{226}Ra, ^{210}Pb and ^{230}Th, the accumulation increased after 14 days and then decreased at 56 days of exposure. The reason why there is a peak in earthworm body concentrations of these radionuclides, followed by a decline at later time points remains unclear and requires further study.

Metals and radionuclides have shown to cause mortality (Spurgeon and Hopkin, 1996a; Janssen et al., 1997) and reduce fertility (Cikutovic et al., 1993; Alonzo et al., 2006), cocoon production (Spurgeon and Hopkin, 1996a; Alonzo et al., 2006), cocoon viability (Spurgeon and Hopkin, 1996b; Alonzo et al., 2006) and growth (Gestel et al., 1991; Khalil et al., 1996) of earthworms. In this study the contaminated soil may have had a significant effect on earthworm's fitness (Fig.13), (Table A2, please see Supplementary Material section), negatively affecting growth, by causing earthworm's weight loss (Fig. 14). A study performed by our team (Niemayer et al. personal communication), also showed that this soil had a negative effect on *Eisenia andrei* and *Enchytraeus crypticus* reproduction, regarding the offspring hatched from the cocoons. The organisms exposed to LUFA 2.2 also suffered weight loss (Fig. 14). This weight loss may be due to the type of food used in this experiment and not the exposure to LUFA 2.2. In similar experiments performed by our team (data not shown), where earthworms were exposed "*in situ*" to the same soils during 56 days, but fed with oat meal instead of dried horse manure, organisms exposed to the soil B showed weight loss, in opposition to a weight gain in organisms exposed to LUFA 2.2.

One of the aims of the present study was to evaluate the usefulness of a genotoxicity assay, such as the comet assay, to assess the level of DNA damage caused by earthworm's exposure to a soil contaminated by metals and radionuclides. Since DNA is an important target of environmental stress in both aquatic and terrestrial organisms (Reinecke and Reinecke, 2004), the loss of DNA integrity may determine the induction of mutations and other irreversible toxic effects such as the "genotoxic disease syndrome" of invertebrates (Kurelec, 1993). The level of strand breakage in DNA has been proposed as a sensitive indicator of genotoxicity and an effective biomarker in environmental biomonitoring (Shugart and Theodorakis, 1998; Shugart, 2000). In the present study, DNA integrity of the earthworms was significantly affected by the exposure to the contaminated soil, since the damages in the DNA of coelomocytes were always significantly higher (Table A2, , please see Supplementary Material section) in organisms exposed to this soil than in those exposed to LUFA 2.2 (Fig. 14).

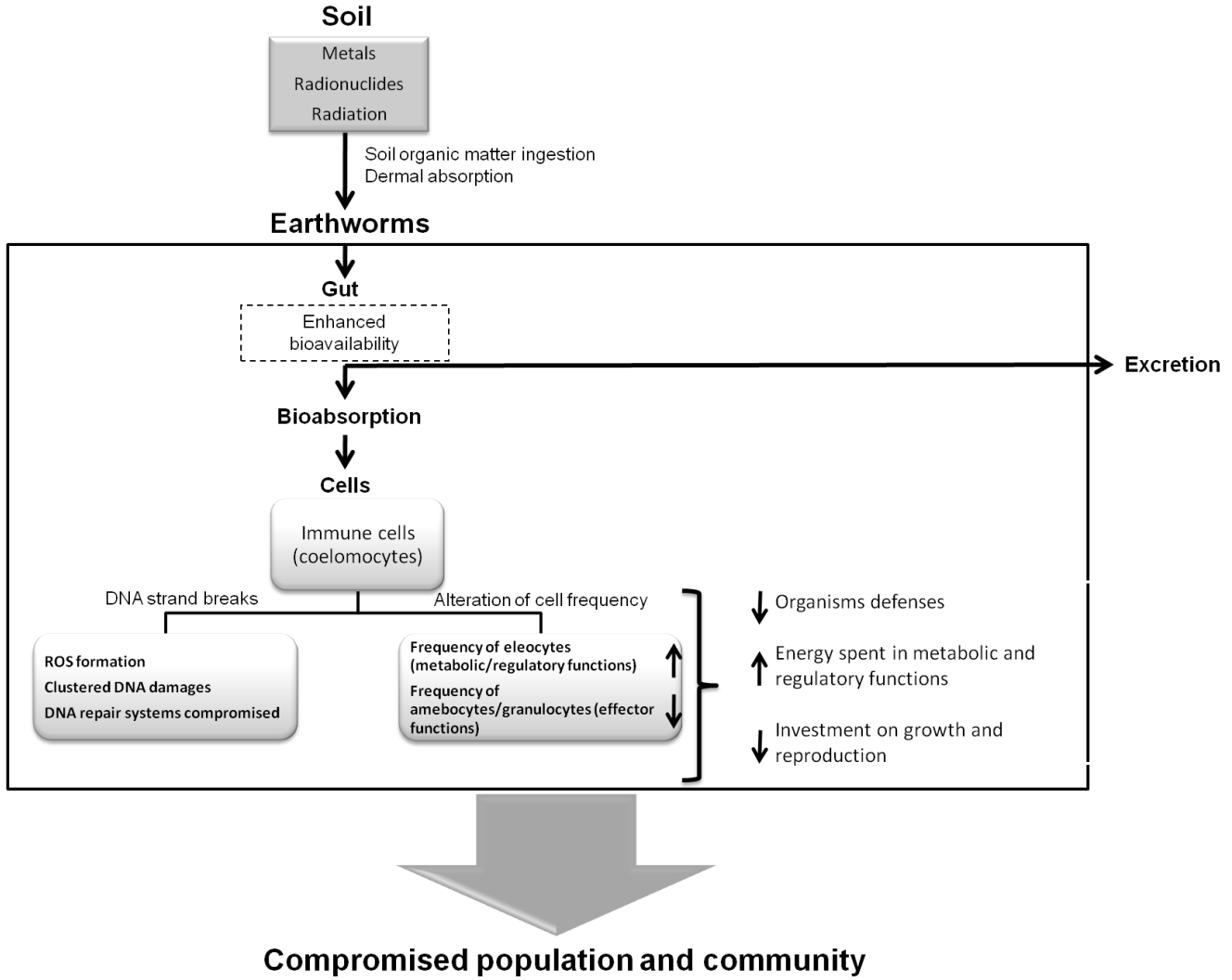

Figure 13: Schematic representation of earthworm's exposure pathway, linking the results obtained in this study and hypothetical effects at higher levels of ecological organization

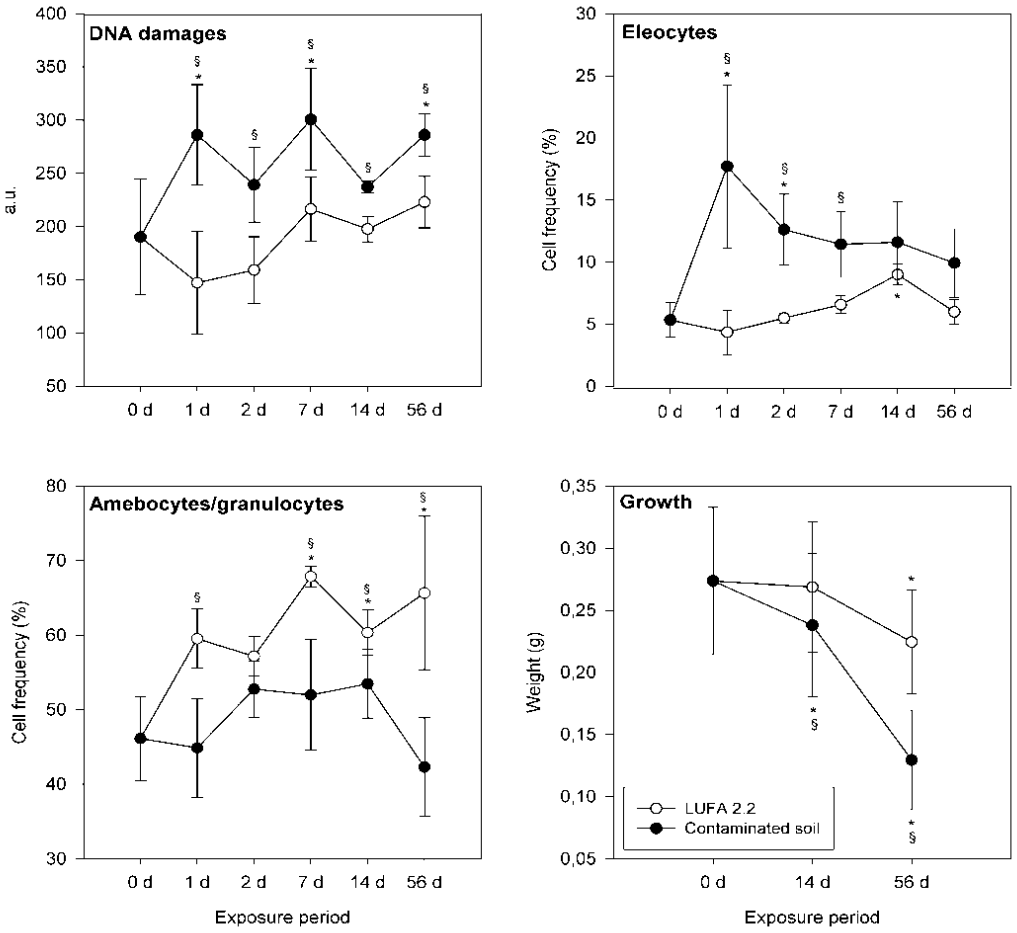

Figure 14: Coelomocytes DNA integrity, eleocytes and amebocytes/granulocytes frequency and growth of *Eisenia andrei* exposed to a contaminated soil (black circles) and to LUFA 2.2 (white circles). Parameters were assessed before exposure (0 d) and after 1, 2, 7, 14 and 56 days of exposure for all analyses, except for growth (before exposure, 14 and 56 days after exposure). Data are shown as average ± standard error. (*) stands for statistical significant differences after a Dunnet's multi-comparison test (p<0.05), to compare exposure periods, of each soil tested, with 0 d. (§) stands for statistical significant differences after a Student's t-test (p<0.05), to compare soils at each exposure period.

These damages were probably caused by the exposure to metals and radionuclides present in this soil as a result of the uranium extraction and processing. As suggested by Barillet and co-workers (2005), some metals induce the production and intracellular accumulation of reactive oxygen species (ROS), that yield DNA damages. Thus, the evaluation of metals sub-lethal toxicity should always include biomarkers of DNA damage (Hartsock et al., 2007). ^{238}U heads the radioactive series that contain the radionuclides ^{234}U, ^{230}Th, ^{210}Po and ^{226}Ra. ^{226}Ra is a radionuclide

with major environmental implications, because it is on the top of an important sub-chain in which are included ^{222}Rn and ^{210}Pb, perhaps the main contributors to the total dose for humans (UNSCEAR, 2000). ^{210}Po is also of great concern since it is highly radiotoxic and an almost pure α-emitter with a half-life of 138.4 days (Carvalho and Oliveira, 2007; Jia et al., 2009). Despite the risk posed by these two radionuclides, all the elements analyzed and bioaccumulated by earthworms, represent serious risks to these species and to all the organisms that are exposed to them, mainly by ingestion, as almost all these particles are mainly alpha-emitters (Carvalho and Oliveira, 2007). The two broad categories of radiation that require consideration in the context of internal dosimetry are photons and charged particles, the latter including electrons and alpha particles (Harrison and Day, 2008). Alpha particles (high energy, relatively large mass and momentum, low velocity) have relatively high linear energy transfer (LET) values, whilst electrons (generally lower energy than alpha particles but far lower mass and therefore greater velocity) have, in general, relatively lower LET values (Harrison and Day, 2008). Clustered DNA damage, together with the degree of complexity of the damage, has been shown to increase with LET (Valentin, 2003). The ultimate biological consequence is dependent on whether the damage can be repaired and with what fidelity (Harrison and Day, 2008). Types of clustered damage may compromise DNA repair fidelity and can lead to an increase in mutation frequency (Pearson et al., 2004).

Flow cytometry was used to evaluate the potential cytotoxic effects caused by the exposure to metals and radionuclides. After the characterization of *Eisenia andrei* coelomocytes, it became clear that these cells were composed mainly by three different populations (Fig. A1, please see Supplementary Material section), which is in agreement with what has been described in the literature (e.g. Adamowicz, 2005; Kurek et al., 2007) for other oligochaetes such as *Dendrobaena veneta* and *Lumbricus terrestris*. Results suggest that earthworm's immune system was affected by the exposure to the contaminated soil (Fig. 14), since there were significant differences between the soils tested (Table A2, please see Supplementary Material section). A higher frequency of eleocytes was observed in the organisms exposed to the contaminated soil (Fig. 14). Eleocytes are cells that participate in various vital processes related with metabolic and regulatory functions, ensuring proper functioning of the whole organism and also in the accumulation of contaminants like metals (Affar et al., 1998; Adamowicz, 2005). Other cells have also important roles such as, amebocytes and granulocytes, which are cells with effector functions like phagocytosis, encapsulation, nodulation and humoral immune responses (Adamowicz, 2005). A lower frequency of these cells was recorded in the organisms exposed to the contaminated soil, during all exposure periods (Fig. 14). The exposure to metals (among other contaminants) has

been shown to be implicated in causing immunological changes in invertebrate species (Galloway and Depledge, 2001). Metals can also compromise immune cell viability and effector functions (Galloway and Depledge, 2001; Homa et al., 2003), making earthworms more vulnerable to external factors. Previous data, reported by Geras'kin et al. (2007) suggest that combined exposure to metals and radionuclides may cause substantial biological effects. Radionuclides can alter immune responsiveness of a variety of animal species (including humans) at exposure levels below which, toxicity endpoints do not respond (Giulio and Hinton, 2008). The effects of ionizing radiation on the immune system can be accessed by estimating changes in cell number or by using a variety of functional assays (UNSCEAR, 2006). The radionuclides of uranium and thorium decay series can act as toxic chemicals for the immune system (Sheppard et al., 2005; UNSCEAR, 2006) inducing stimulatory or suppressive effects (Geras'kin et al., 2007). High doses of radiation produce immune suppression mainly due to the destruction of cells. At low doses and dose rates, the effects may be suppressive or stimulatory (UNSCEAR, 2006). In this study we are possibly looking at both suppressive and stimulatory effects, related with the decrease in amebocytes/granulocytes number and with the increase of eleocytes. However, the increase of eleocytes, which are cells with metabolic and regulatory properties, may also be a response/adaptation of earthworms to the soil contamination. We also have to keep in mind that this soil displays a complex mixture of contaminants, whose interactive effects are unknown (Antunes et al., 2008a).

2.5 Conclusions

In different integrated studies, attempts have been made to correlate the comet assay response with other sub-lethal parameters such as cytotoxicity (Jha, 2008). As expected, these important sub-lethal responses would influence the overall fitness including reproductive success of the organisms (Jha, 2008). In general, it has been suggested that comet response (DNA damage) can be linked to effects on growth, development and reproduction in a variety of organisms and could be used as an early warning biomarker for toxicant exposure on the populations (Lee et al., 2000). Our results show that comet assay and flow cytometry are useful techniques to evaluate the toxicity of contaminants such as metals and radionuclides in earthworms. We also showed that DNA strand breakage and immune cells frequency are important endpoints to be used in the earthworm reproduction assay, for the evaluation of soil toxicity. Together, these results show that the comet assay can be easily included in studies to evaluate the risk of exposure to compounds or environmental matrixes that potentially have

genotoxic properties, namely metals and radionuclides. In particular, this is the first study that integrates DNA damage, cytotoxicity evaluation, growth and bioaccumulation of metals and radionuclides, in earthworms exposed to soil from an abandoned uranium mine.

2.6 Supplementary Material

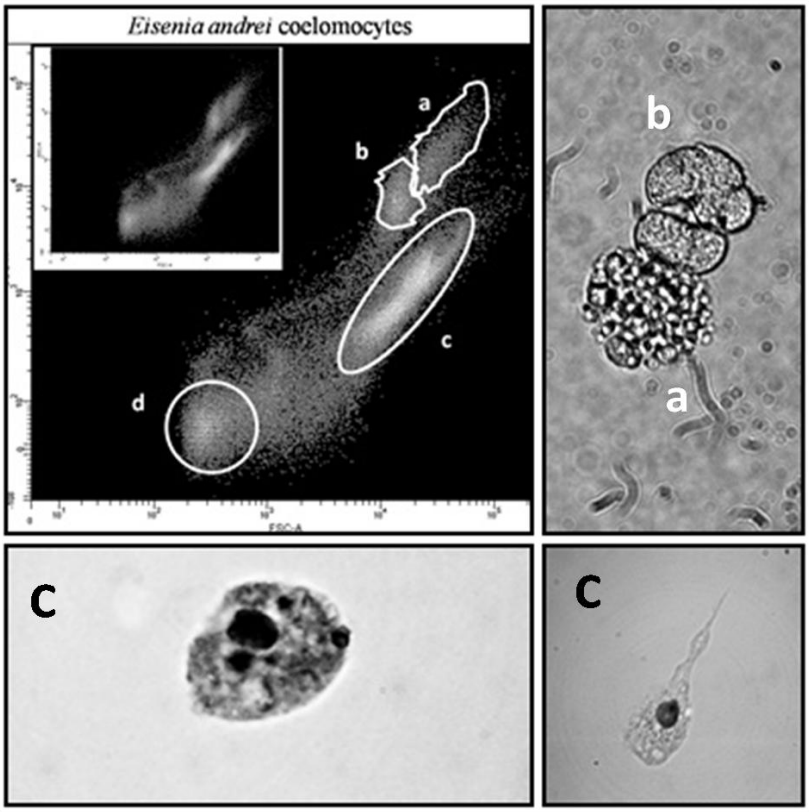

Figure A 1: Coelomocytes of *Eisenia andrei*: a – eleocyte, 1000x; b – immature eleocyte, 1000x; c – granulocyte, 1000x; c – amebocyte 1000x; d – cell debris; separated through cell sorting according to their size and complexity (first image on the left).

Table A 1: Summary table of the two-way ANOVA applied to metals evaluated in *Eisenia andrei* exposed to contaminated soil from an abandoned uranium mine (d.f. – degrees of freedom; *p* – probability).

Parameter	Source of variance	F	d.f.	*p*
Be	Time	51.174	5	<0.001
	Soil	1110.02	1	<0.001
	Time x Soil	47.901	5	<0.001
Al	Time	3.433	5	0.01
	Soil	124.432	1	<0.001
	Time x Soil	7.056	5	<0.001
Mn	Time	19.787	5	<0.001
	Soil	490.860	1	<0.001
	Time x Soil	21.810	5	<0.001
Fe	Time	1.555	5	NS
	Soil	59.685	1	<0.001
	Time x Soil	3.890	5	0.005
Ni	Time	11.159	5	<0.001
	Soil	474.243	1	<0.001
	Time x Soil	21.656	5	<0.001
Zn	Time	16.596	5	<0.001
	Soil	270.682	1	<0.001
	Time x Soil	12.970	5	<0.001
Se	Time	37.266	5	<0.001
	Soil	4.619	1	0.038
	Time x Soil	2.702	5	0.036
Sr	Time	2.918	5	0.022
	Soil	13.127	1	<0.001
	Time x Soil	2.657	5	0.034
Cd	Time	5.814	5	<0.001
	Soil	2.381	1	NS
	Time x Soil	0.992	5	NS
Ba	Time	1.723	5	0.147
	Soil	127.72	1	<0.001
	Time x Soil	5.687	5	<0.001
Pb	Time	3.826	5	0.006
	Soil	53.247	1	<0.001
	Time x Soil	3.459	5	0.01
U	Time	150.24	5	<0.001
	Soil	3296.94	1	<0.001
	Time x Soil	133.670	5	<0.001

Table A 2: Summary of the two-way ANOVA applied to growth, DNA integrity (comet assay) and immune cells frequency (flow cytometry), evaluated in *Eisenia andrei* exposed to contaminated soil from an abandoned uranium mine (d.f. – degrees of freedom; *p* – probability) and to the natural reference soil LUFA 2.2.

Parameter	Source of variance	F	d.f.	p
Growth	Time	120.403	2	<0.001
	Soil	51.721	1	<0.001
	Time x Soil	26.547	2	<0.001
Comet assay	Time	3.566	5	0.012
	Soil	27.696	1	<0.001
	Time x Soil	5.052	5	0.004
Flow cytometry (Eleocytes)	Time	4.686	5	0.002
	Soil	68.435	1	<0.001
	Time x Soil	8.385	5	<0.001
Flow cytometry (Amebocytes/Granulocytes)	Time	4.227	5	0.005
	Soil	37.071	1	<0.001
	Time x Soil	3.958	5	0.007

NS: non-significant (P>0.05)

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CHAPTER 3 - HISTOPATHOLOGICAL CHANGES IN THE EARTHWORM *EISENIA ANDREI* ASSOCIATED WITH THE EXPOSURE TO METALS AND RADIONUCLIDES

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Lourenço, J., Silva, A., Carvalho, F., Oliveira, J., Malta, M., Mendo, S., Gonçalves, F., Pereira, R., 2011. Histopathological changes in the earthworm *Eisenia andrei* associated with the exposure to metals and radionuclides. Chemosphere 85, 1630-1634.

3.1 Abstract

Earthworms *were* exposed for 56 days to a contaminated soil, from an abandoned uranium mine, and to the natural reference soil LUFA 2.2. Histological changes in earthworm's body wall (epidermis, circular and longitudinal muscles) and gastrointestinal tract (chloragogenous tissue and intestinal epithelium) were assessed, after 0, 14 and 56 days of exposure. Results have shown alterations in all the studied tissues after 14 days of exposure (except for the intestinal epithelium), yet more severe effects were registered after 56 days of exposure. Herein we report histopathological alterations as a good biomarker for the evaluation of soil quality. We also demonstrate that morphological changes in the body wall and gastrointestinal tract, are important endpoints that could be added to earthworm's standardized tests, for the evaluation of soil toxicity, as part of the risk assessment of contaminated areas.

Keywords: *Eisenia andrei*; histopathology; metals; radionuclides.

3.2 Introduction

Uranium is the heaviest naturally occurring element with both radiotoxic and chemotoxic properties (Hartsock et al., 2007). Uranium can enter the environment through several human activities such as, nuclear industries and mining (Craft et al., 2004). The surroundings of these facilities may become contaminated by routine releases or accidental leaks or spills from the various physical and chemical processes carried out to extract and treat the ore (Giovanetti et al., 2010). In Portugal, the uranium extraction was performed mainly by underground, open pit mining and in situ-leaching, to recover uranium from the poorest ore (Pereira et al., 2004a; Antunes et al., 2008a; Pereira et al., 2008; Carvalho et al., 2009a). In several areas, uranium mining produced large amount of wastes, with significant amounts of chemical and radioactive elements (Pereira et al., 2004b; Antunes et al., 2008a; Pereira et al., 2008). The exposure of wastes to geodynamic processes has promoted the transfer of these elements to the different environmental compartments (Pereira et al., 2004a), where they have probably become bioavailable gaining potential to impact edaphic communities.

Earthworms are important organisms of soils ecosystems, playing a major role in the comminution and mineralisation of organic matter and thus greatly influencing soil structure and chemistry (Edwards and Bohlen, 1996). The importance of earthworms to test the potential adverse effects of chemicals on soil organisms has been recognized by various environmental organizations and resulted in a set of standard test guidelines involving earthworm species (OECD, 1984, 2004; ISO, 2005). The use of biomarkers is becoming increasingly important in the evaluation of the effects of contaminants in earthworms. The need for developing new biomarkers for these organisms, to complement responses usually evaluated, by standard toxicity tests, has been discussed (Reinecke and Reinecke, 2004; Gastaldi et al., 2007).

The term “biomarker” or “biomarker response” often refers to cellular, biochemical, molecular, or physiological changes that are measured in cells, body fluids, tissues, or organs within an organism and are indicative of xenobiotic exposure and/or effect (Peakall, 1992; Lam and Gray, 2003). Because of their relatively immediate response, these biomarkers have also been proposed as “early warning” tools for biological effect measurement (Peakall, 1992; Cajaraville et al., 2000; Lam and Gray, 2003; Amaral et al., 2006). Biological changes caused by contaminants are referred as “effect biomarkers” (Lam and Gray, 2003; Van Der Oost et al., 2003). Thus, histological alterations in tissue , which can significantly modify the function of tissues and cells, may signal damaging effects in organisms, resulting from prior or ongoing exposure to toxic agents (Reddy and Rao, 2008). Histopathological alterations are mid-term responses to sublethal

stressors that have been used as valuable tools to evaluate the toxic effects of contaminants (Lam and Gray, 2003). The histological changes observed depend on the ability of the organisms to repair the injury, the nature and severity of the contamination and the length of the exposure (Haschek and Rousseaux, 1998). Histopathological responses in earthworms have been reported as valuable markers of toxicity in previous studies (Amaral et al., 2006; Giovanetti et al., 2010; Kiliç, 2011).

Here we report the histopathological changes observed in earthworms (*Eisenia andrei*) exposed to soils contaminated with uranium mining wastes containing high levels of metals and radionuclides.

3.3 Materials and Methods

3.3.1 Soils tested

The standard natural soil LUFA 2.2 (Speyer, Germany), was used as the control soil. The test soil [(soil B), according to Pereira et al. (2008)] consisted of a contaminated soil from the Cunha Baixa uranium mine (Mangualde, Centre of Portugal). The high contamination of soil B mainly results from the deposition of sludge from an effluent treatment plant (Antunes et al., 2008b), which is still operating. It displayed high concentrations of metals (namely U, Mn, Al and Sr) and radionuclides (Table 5) and high toxicity for the earthworm *Eisenia andrei* (Antunes et al., 2008b) and terrestrial plants (Pereira et al., 2009). After discarding the superficial layer (plant debris), the first 20 cm of soil were collected and sieved to discard the >2 mm fraction. Soil was collected in three different points and mixed to obtain a composite soil sample for the site. Prior to the test, pH, water content (%) and water holding capacity (WHC) of both soils were measured and soil water content was adjusted to 40% of WHC_{max}. Soil pH was measured in a soil – water suspension (1:5 w/v extraction ratio) according to the method described in FAOUN (1984). Soil water content was determined from weight loss after drying the soils at 105 °C, for 24 h. Water holding capacity (WHC) of soils was determined as described in ISO (1998).

Table 5: Main physico-chemical characteristics, metal and radionuclide concentrations in both the contaminated soil (soil B) and the reference LUFA 2.2 soil

	Lufa 2.2	**Soil B**
pH	5.89 ± 0.26	7.79 ± 0.01
Conductivity (µS/cm)	49.17 ± 2.94	2263 ± 11.55
Moisture (%)	2.28 ± 0.81	48.2 ± 0.12
Organic matter (%)	3.61 ± 0.33	7.71 ± 0.60
Metals (µg/g soil)		
Al	3656.33 ± 196.09	26440 ± 1109.77
Ba	48.21 ± 6.93	8.50 ± 14.04
Be	0.34 ± 0.03	50.11 ± 4.29
Cd	0.09 ± 0.01	2.58 ± 0.23
Fe	3345 ± 635.24	13383.33 ± 654.44
Mn	119.38 ± 7.83	3711.33 ± 103.27
Ni	2.62 ± 0.39	91.38 ± 1.48
Pb	10.26 ± 0.32	9.72 ± 0.71
Sr	8.68 ± 0.12	19.29 ± 12.41
U	1.08 ± 0.49	215.72 ± 8.50
Zn	13.01 ± 0.52	511.73 ± 4.94
Radionuclides (Bq/Kg soil)		
^{238}U	19.2 ± 1.0	3696 ± 101
^{235}U	0.78 ± 0.17	163 ± 6
^{234}U	23.4 ± 1.2	3617 ± 98
^{230}Th	115 ± 8	10682 ± 335
^{226}Ra	20 ± 6	1506 ± 182
^{232}Th	20.6 ± 3.3	24.0 ± 2.1
^{210}Pb	21.2 ± 1.1	2318 ± 214

Adapted from Lourenço et al. (2011) (for further details please see Pereira et al. (2008) and André et al.(2009))

3.3.2 Test organism

E. andrei used for the assay were obtained from a synchronized laboratorial culture, reared in large containers, under controlled temperature 20±2 °C and photoperiod (16 h^L: 8 h^D) conditions. A mixture composed by peat moss, horse manure and $CaCo_3$ (to adjust the pH between 6 and 7) is the substrate used to maintain the laboratorial cultures. This substrate is periodically moistened and monitored for pH. Each fifteen days, new defaunated horse manure is added to guarantee the supply of food to earthworms.

3.3.3 Laboratory exposure

Organisms were exposed to both soils in 1-L plastic buckets (12 cm height and 10 cm in diameter), with lids bearing one opening at the top as described by Antunes et al. (2008a). The openings were covered with 300 μm nylon mesh, using white thermal glue (supplied by Elis–Taiwan, Taiwan, ref. TN122/WS), which has been shown to be non-toxic to aquatic invertebrates (Pereira et al., 2000). Before exposure, the organisms were acclimated for 24 h in a container with LUFA 2.2 soil.

For the present study, 5 chambers were used for each soil tested and sampling periods (14 and 56 days), making a total of 20 chambers. Each chamber contained 10 earthworms. After 14 and 56 days of exposure, earthworms were removed from the 5 chambers of each soil (a total of 50 animals), and 5 of them were used for histopathological analysis. The remaining animals were used to measure other parameters (data not shown).

Animals were sampled before exposure (0 days), directly from the acclimation containers with LUFA 2.2 soil. According to international standard guidelines (ISO, 1998; OECD, 2004), adult earthworms with clitellum and body mass between 250 and 600 mg were used.

During the experiment, organisms were fed, once a week, with 5 g *per* test chamber of horse manure. The experiment was carried out using the temperature and photoperiod described for culture maintenance.

3.3.4 Analysis of metals and radionuclides

Total metal concentrations in the test and control soil were determined by André and co-authors (2009).

The analyses of radionuclides on soil samples were performed according to the methodology described by Carvalho et al. (2007; 2009b).

3.3.5 Analysis of histological alterations

At each sampling period, earthworms were removed from the soil and washed with distilled water. After a depuration of 6 hours, as recommended by Maenpaa et al. (2002) and applying the methodology described by Muthukaruppan et al. (2005), to release gut contents, earthworms were narcotized (placing them in 7% alcohol until relaxed), transversely cut in 3 parts and fixed in Bouin solution for 24 h. Earthworms were sequentially dehydrated in graded ethanol

concentrations and embedded in paraffin wax with different melting points. Slides with transverse sections of earthworms (7 μm thick) were stained using the hematoxilin-eosin method for light microscope observation, and photographed with an Olympus CKX41 microscope equipped with a digital color camera Olympus SC30.

3.4 Results and Discussion

In the present study, extensive alterations were observed in the tissues of the body wall and intestinal tract of the organisms exposed to the mining soil (soil B) (Fig. 15 and 16). After 14 days of exposure, it was possible to observe, that the epidermis has suffered damages, showing large intercellular spaces, and loss of structural integrity, when compared with that of organisms exposed to LUFA 2.2. (Fig. 15).

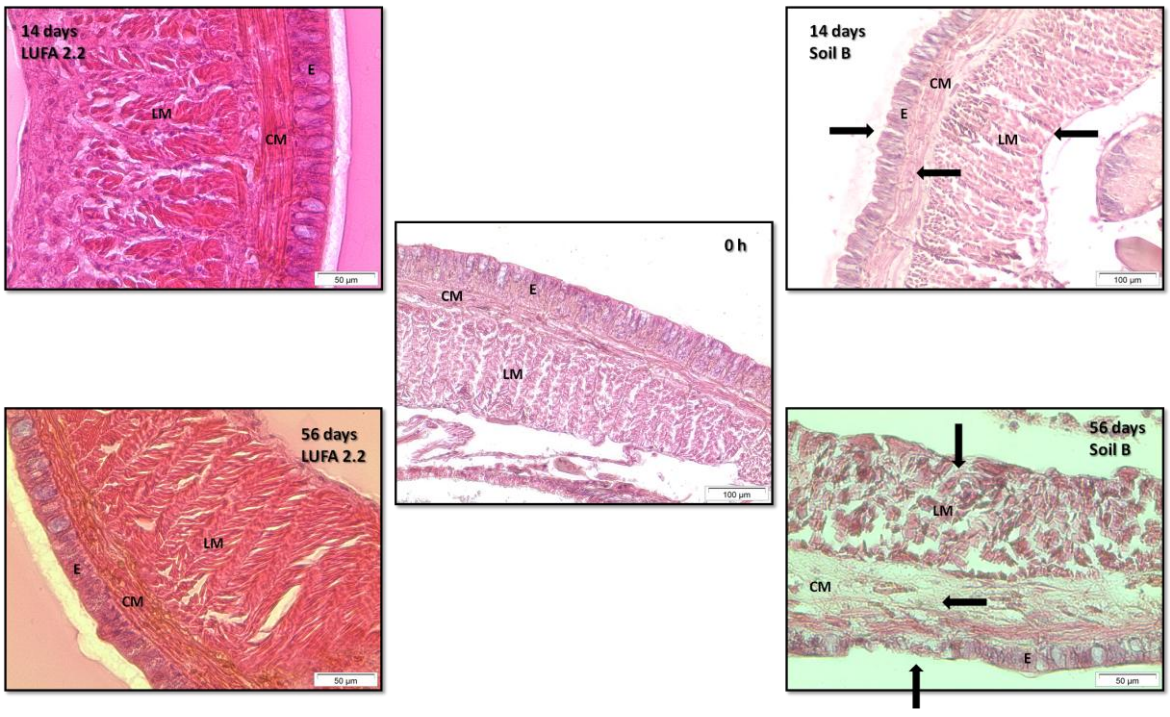

Figure 15: Transverse sections of *E. andrei*, showing epidemis (E), circular muscle (CM) and longitudinal muscle (LM) in the corresponding exposure period (0 h, 14 and 56 d) in both soils (LUFA 2.2 – control soil; soil B – contaminated soil). 14 d Soil B – intercellular spaces and loss of structural integrity in the epidermis; deformation of the circular and longitudinal muscles. 56 d Soil B – deep signs of degradation and necrosis of the epidermis; deformation and extended necrosis of the circular muscle layer; atrophy and complete alteration of the structural integrity of the longitudinal muscle layer.

The circular muscle also evidenced loss of cell shape and organization of the muscle fibers and necrosis (restricted to some regions) (Fig. 15). The same alterations were observed in the longitudinal muscle layer, but were less evident (Fig. 15). After 56 days of exposure, the epidermis displayed severe signs of degradation and necrosis, almost exposing the circular muscle (Fig. 15). Some regions showed an almost complete disintegration of the epithelial cell lining (Fig.15). The circular muscle showed extensive loss of structural organization and loss staining properties, when compared to that of organisms exposed to LUFA2.2 (Fig. 15). This muscular layer was almost completely destroyed, suffering extensive necrosis (Fig. 15). The longitudinal muscle displayed atrophy and was also completely altered in its structural integrity (Fig. 15). Cells showed necrosis and severe changes on their shape and, consequently, muscle fibers were completely disintegrated. The histological changes observed in earthworm's body wall may have been caused by the exposure to metals and radionuclides available in the soil solution, since one of the main exposure routes of earthworms is through dermal contact (Vijver et al., 2005; Hobbelen et al., 2006). In fact, several studies have reported the presence of metals in earthworm's body wall (Morgan and Morgan, 1998; Prinsloo et al., 1999; Kiliç, 2011). Kiliç et al. (2011) observed that metals were found to cause damage and to be accumulated mainly in the circular and longitudinal muscles of earthworms exposed to polluted soils. Therefore, it seems feasible that this may also be the case, since major alterations and damages were observed in the tissues of earthworms exposed to soil B after 14 and 56 days. In fact, in a previous work (Lourenço et al., 2011) conducted in parallel with this one, our team observed that almost all the metals analyzed (Be, Al, Ba, Mn, Fe, Ni, Zn, U) and also all the radionuclides (except for ^{232}Th), were found in significant higher concentrations in the earthworms exposed soil B, when compared to those from the Lufa 2.2 soil. The concentration of most of the metals analyzed, in the organisms exposed to soil B, have increased after 7 days of exposure and started to decrease till the end of the experiment (day 56). However, the metals concentrations in organisms exposed to soil B were always significantly higher than those in organisms exposed to LUFA 2.2 (both after 14 and 56 days of exposure). Radionuclides behavior was somewhat different, since the concentrations of most of these continued to increase till the end of the experiment (except for ^{230}Th, ^{226}Ra and ^{210}Pb), revealing earthworms difficulties in dealing with these elements. Although taxonomically distant, various studies performed on fish also reported necrosis in muscles and histological alterations of the epidermis, due to metals and uranium exposure (Vinodhini and Narayanan, 2009; Barillet et al., 2010; Poleksic et al., 2010).

Earthworm's chloragogenous tissue and intestinal epithelium also revealed histopathological changes when organisms were exposed to soil B (Fig. 16). After 14 days of exposure, the chloragogenous tissue presented clear changes, as cells began to lose their shape, while no major effects were observed in the intestinal epithelium (Fig. 16). After 56 days of exposure, the chloragogenous tissue showed signs of severe changes and necrosis (Fig. 16). Cells completely lost their shape, compromising the structural integrity of the chloragogenous tissue.

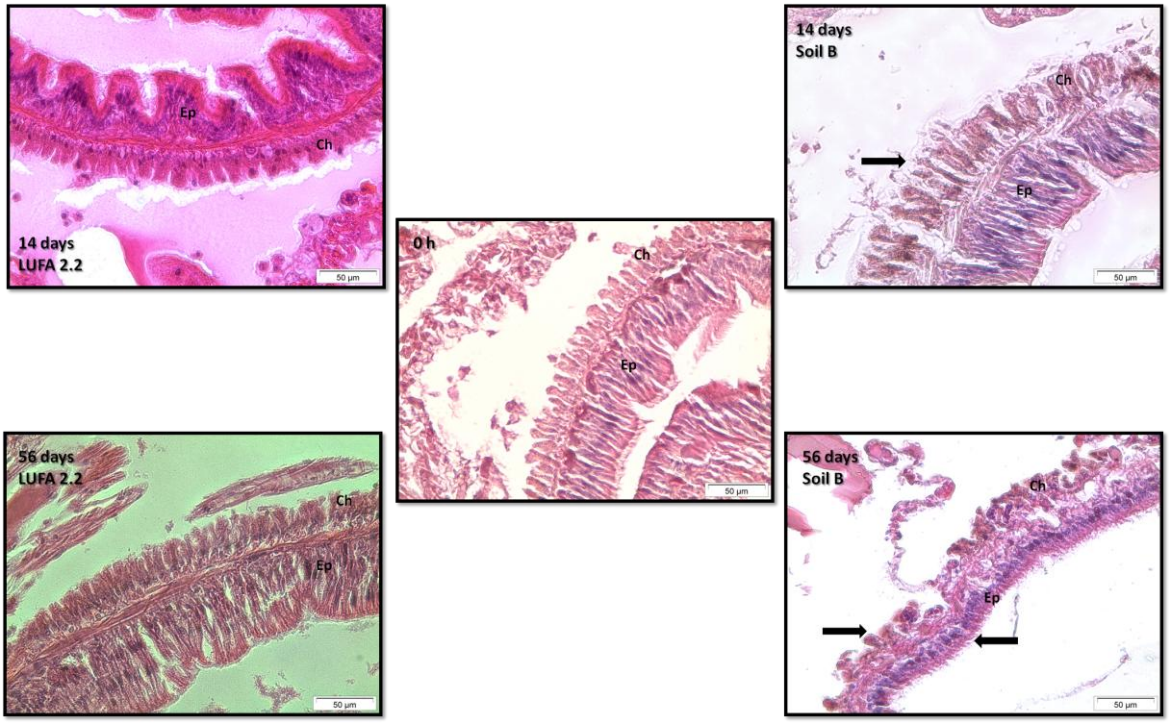

Figure 16: Transverse sections of *E. andrei,* showing intestinal epithelium (Ep) and chloragogenous tissue (Ch) in all exposure periods (0 h, 14 and 56 d) in both soils tested (LUFA 2.2 – control soil; soil B – contaminated soil). 14 d Soil B – some deformation of the chloragogenous tissue. 56 d Soil B – severe degradation and necrosis of the chloragogeneous tissue; reduction of the thickness of the intestinal epithelium suggesting serious atrophy.

The atrophy of the intestinal epithelium was evident, as it became very thin, when compared to that of earthworms exposed to LUFA 2.2. The intervilli spaces disappeared and the villi were fused, forming a continuous and thin layer of cells (Fig. 16). Similar damages were also observed by Kiliç et al. (2011) on these tissues. The chloragogenous tissue (sheath of modified peritoneal cells), located in the gastrointestinal canal and separating the absorptive epithelium from the coelom, constitutes the main site of metals accumulation including uranium and depleted

uranium (Morgan et al., 2002; Giovanetti et al., 2010). According to Morgan et al. (2002), morphological alterations in the earthworm chloragogenous tissue are a way of handling larger quantities of metals. The elimination of these contaminants may be achieved through the extrusion of whole chloragocytes (Cancio et al., 1995), which enable earthworms to tolerate high concentrations of metals in the soil, at least to a certain point (Stürzenbaum et al., 1998; Langdon et al., 1999; Langdon et al., 2001). Morgan et al. (2002) also observed that the intestinal epithelium of the oligochaete *Dendrodrilus rubidus* has also a great ability for metals accumulation, and this seems also to be the case of the morphological changes herein observed and reported.

The results herein reported, reinforce previous genotoxic and cytotoxic effects observed in earthworms exposed for 56 days to a contaminated soil from the Cunha Baixa uranium mine (Mangualde, Portugal) (Lourenço et al., 2011).

3.5 Conclusions

To the best of our knowledge this is the first study reporting histopathological alterations in earthworms caused by the exposure to metals and radionuclides simultaneously. Together, this study and another one recently published by our group (Lourenço et al., 2011), show that soils from abandoned uranium mining areas contaminated with metals and radionuclides pose serious risks to the overall fitness and survival of epigeic earthworms populations and communities. Moreover, these studies show that it is possible to evaluate bioaccumulation, molecular and histological endpoints along with mortality, growth and reproduction in a single sub-lethal test with earthworms, reducing the uncertainty of the information provided by the ecotoxicological line of evidence in the risk assessment process of contaminated areas. Strong effects were observed at the tissues level, mainly muscular and chloragogenous tissue, in parallel with the bioaccumulation of metals and radionuclides, and with other alterations at the molecular and individual level. Thus, the evaluation of histological changes in the body wall and gastrointestinal tract can be used as reliable and sensitive biomarkers of harmful effects yielded by available metals and radionuclides, in earthworms exposed to uranium mining residues and other radioactive wastes. The information provided by these biomarkers combined with those provided by the analysis of molecular biomarkers could improve our ability to understand the mechanisms of toxicity underlying effects at the individual level, like growth and reproduction impairments.

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CHAPTER 4 - EVALUATION OF THE SENSITIVITY OF GENOTOXICITY AND CYTOTOXICITY ENDPOINTS IN EARTHWORMS EXPOSED *IN SITU* TO URANIUM MINING WASTES

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4.1 Abstract

Earthworms were exposed for 56 days to a contaminated soil from an abandoned uranium mine and to the natural reference soil LUFA 2.2. The exposure occurred *in situ*: the containers with contaminated soil were placed near the mine pit; the containers with reference soil were placed in a reference site. For the assessment of metals bioaccumulation, DNA damages, cell-to-cell variation in DNA content, Median Fluorescence Intensity (MFI), coelomocytes frequency and proliferation, organisms were sampled after 0, 1, 2, 7, 14 and 56 days of exposure. For the assessment of radionuclides bioaccumulation, animals were sampled after 0, 14 and 56 days of exposure. As for growth, organisms were sampled after 0, 14, 28 and 56 days of exposure. The reproduction assay was performed according to the OECD (2004) guideline. DNA damages were assessed by comet assay and flow cytometry was used to determine cell-to-cell variation in DNA content, Median Fluorescence Intensity (MFI), coelomocytes frequency and proliferation. Results have shown a myriad of effects in the organisms exposed to the contaminated soil, namely: the inhibition of reproduction, growth reduction, DNA damages, cytotoxicity, changes in eleocytes fluorescence intensity, coelomocytes proliferation and bioaccumulation of metals and radionuclides. Our results showed that the evaluation of genotoxicity and cytotoxicity endpoints, along with other parameters at an individual level, in standard reproduction assays conducted *in situ,* are important to improve the risk assessment process of areas contaminated with uranium and other radioactive mining wastes.

Keywords: *Eisenia andrei*; DNA damages; metals; radionuclides; cytotoxicity

4.2 Introduction

The exploitation of radioactive ores in Portugal lasted from 1908 to 2001, and took place mainly in the north-central part of the country (Carvalho et al., 2009a). The uranium mining industry generated considerable volumes of wastes often containing high concentrations of toxic metals and radionuclides (Antunes et al., 2008a). Human and other living organisms, from uranium mining districts may be exposed to radiation doses from mining, milling, transport of radioactive wastes, radioactive dust and contaminated water and foodstuffs (IAEA, 2005). The risks of such wastes are usually determined through chemical analyses of environmental samples. Nevertheless, soil toxicity cannot be evaluated only through chemical analyses, since it is becoming clear that it does not consider mixture toxicity and the role of soil properties, in determining chemicals bioavailability (Gastaldi et al., 2007). The best integrators of these complex effects are the exposed organisms themselves (Gastaldi et al., 2007).

Toxicity assays conducted under controlled laboratory conditions, lack the integration of natural fluctuating environmental conditions (Moreira et al., 2005). Consequently, it is difficult to extrapolate laboratory results to field situations. One critical issue regarding laboratory assays is understanding to what extent they reflect the actual field toxicity, and whether, as a result, the assays generate ecologically relevant information for the area under study (Moreira et al., 2005). By integrating natural fluctuating environmental conditions, *in situ* assays allow more realistic exposures (Moreira et al., 2005). Many examples are available for *in situ* assays in the aquatic compartment (water and sediment), showing their successful application to environmental contamination scenarios (e.g. Castro et al., 2004; Moreira et al., 2005). In contrast, less attention has been given to soils, which is reflected in the scarce published studies or standard protocols on *in situ* assays (e.g. Antunes et al., 2008a; ISO, 11268-3: 1999). Thus, it is important to develop methodologies for the soil compartment analysis in order to provide tools to allow more realistic assessments of soil toxicity (Antunes et al., 2008a).

Earthworms are important members of the terrestrial ecosystems, contributing to soil aeration, maintenance of soil permeability and structure, as well as for the degradation of organic matter, consuming large quantities of soil in the process (Button et al., 2010). The importance of earthworms in testing the potential adverse effects of chemicals on soil organisms has been recognized by various environmental organizations and resulted in the elaboration of a set of standard test guidelines (OECD, 1984, 2004; ISO, 17512-1:2008). Even though these bioassays produce relevant data, additional information is needed, concerning the biological effects of contaminants on other sub-lethal endpoints, in order to identify the mechanisms of

action of contaminants in the ecosystem (Antunes et al., 2008a). Standard toxicity tests (mortality and reproduction rates) can be complemented by the use of biomarkers, which can provide more information about the organism's stress response to individual toxicants and mixtures (Gastaldi et al., 2007). The use of biomarkers is becoming increasingly important in the evaluation of the effects of contaminants in earthworms and the need to develop new biomarkers for these organisms, to supplement other endpoints in standard toxicity tests, has been largely discussed (Spurgeon et al., 2003; Reinecke and Reinecke, 2004; Martin et al., 2005; Gastaldi et al., 2007; Bonnard et al., 2009).

This study aimed to evaluate the applicability of the earthworms reproduction assay (OECD, 2004) and the determination of the genotoxicity and cytotoxicity effects, in earthworms exposed *in situ* to uranium mining wastes. Uranium, the heaviest naturally occurring element, has radiotoxic and chemotoxic properties and it is both a genotoxic and cytotoxic agent (Hartsock et al., 2007). These characteristics highlight the importance of the assessment of genotoxicity and cytotoxicity endpoints, in studies involving the exposure to uranium and its daughter radionuclides. To that end, the evaluation of biomarkers of genotoxicity (DNA damages, cellular DNA content) and cytotoxicity (cell frequency, median fluorescence intensity, cell proliferation), in earthworm's coelomocytes, was additionally considered in this assay. Simultaneously, the bioaccumulation of metals and radionuclides by earthworms was also assessed, to clarify cause-effect relationships.

4.3 Materials and Methods

4.3.1 Study site

The Cunha Baixa uranium mine, near the city of Viseu, Portugal, is one of the largest uranium mines in the country (Carvalho et al., 2009a). The exploitation of the ore began through underground mining, but later evolved to open-pit extraction (1970 until 1993) (Antunes et al., 2008a; Pereira et al., 2008; Carvalho et al., 2009a). Sulfuric acid was used for in situ leaching of uranium in underground works as well as in heap leaching of low grade ores at the surface (Antunes et al., 2008a; Pereira et al., 2008; Carvalho et al., 2009a). Since mining had stopped, the acid water from the mine pit has been pumped out to waste water treatment plants and treated with burned lime and barium chloride for pH neutralization and precipitation of radionuclides, respectively (Pereira et al., 2008; Carvalho et al., 2009a). When the maximum capacity of the

treatment pond is reached, the water is released to a nearby stream and the sludge formed by the accumulation of the chemical compounds at the bottom is removed and spread near the mine pit. The sludge has high levels of metals (Pereira et al., 2008) and radionuclides from uranium series and low levels of radionuclides from the thorium series (Carvalho et al., 2007). Soils from the surrounding areas of the mine pit have suffered several impacts, through the deposition of both tailings and sludge from the waste water treatment plant (the latter still ongoing) (Antunes et al., 2008a; Pereira et al., 2008).

4.3.2 Soils tested

Previous studies have evaluated the degree of contamination and toxicity profiles of several soils in the Cunha Baixa uranium mine area (Antunes et al. 2008b; Pereira et al. 2008). Considering this information, two sites were chosen for the experiment: site B, used for the deposition of sludge from the waste water treatment plant, with a radiation level of 850 cps and highly contaminated with metals and radionuclides and site I, selected as a reference site due to its relatively low radiation level (414 cps) and distance from the mine pit (Antunes et al. 2008b; Pereira et al. 2008). The soil from site B (soil B), was chosen as test soil, as it displayed high extractable concentrations of metals, namely U, Mn, Al and Sr (Pereira et al. 2008) (please see Table 5 in chapter 3) and high toxicity for invertebrates and plants (Antunes et al. 2008b; Pereira et al. 2009). The standard reference soil LUFA 2.2 (Speyer, Germany) was used as control soil.

After removing the superficial layer (plant debris), the first 20 cm of soil were collected and sieved to discard the >2 mm fraction. Water holding capacity (WHC), water content (%) and pH of both soils were measured and soil water content was adjusted to 40% of WHC_{max}. Water holding capacity (WHC) of soils was determined as described in (ISO 11268-2:1998, 17512-1:2008). Soil water content was determined considering the weight loss after drying the soils at 105 °C, for 24h. Soil pH was measured in a soil–water suspension (1:5 w/v extraction ratio) according to the method described in FAOUN (1984).

4.3.3 Test organisms

The earthworms (*Eisenia andrei*) used for the assay were obtained from a synchronised laboratorial culture, reared in large containers, under controlled temperature conditions (20±2 °C) and a photoperiod 16 h^L: 8 h^D. The substrate is composed by sphagnum peat, horse manure and

$CaCO_3$ (to adjust the pH between 6 and 7), and is periodically moistened with distilled water and the pH monitored. The organisms are fed twice a month with dry and defaunated manure. According to international standard guidelines recommendations (OECD, 2004; ISO, 11268-2:1998), adult earthworms with clitellum and body mass between 250 and 600 mg were used in this study. Before exposure, the organisms were acclimated for 24 h in containers with the soil LUFA 2.2.

4.3.4 *In situ* exposure

Organisms were exposed in 1-L plastic buckets (12 cm height and 10 cm in diameter), with lids bearing two openings, one at the top and another at the bottom, for ventilation (Antunes et al., 2008a). The openings were covered with 300 μm nylon mesh, using white thermal glue (supplied by Elis–Taiwan, Taiwan, ref. TN122/WS), which has been shown to be non-toxic to aquatic invertebrates (Pereira et al., 2000). The containers to be placed in site B were filled with soil B, and the ones to be placed in site I, were filled with LUFA 2.2, to eliminate both the impact of chemical contamination and radiation. The *in situ* assay began with the introduction of 10 earthworms in each container that were placed inside a depression made in each exposure site. The placement of the containers was performed as described by Antunes et al. (2008a).

Forty two containers were used for each soil tested (in a total of 84 containers). Earthworms from 25 containers (*per* soil tested), were used for the comet assay, flow cytometry and metal body burdens analysis (5 containers were sampled for each exposure period: 1, 2, 7, 14 and 56 days). For radionuclides bioaccumulation and reproduction assay, 12 and 5 containers (*per* soil tested) were used respectively. The analyses of radionuclides were performed on pools of 20 earthworms obtained from 6 containers sampled after two exposure periods only: 14 and 56 days. The remaining 5 containers for reproduction endpoints assessment were maintained in the soil for 56 days (adults were removed on the 28^{th} day). The reproduction assay was performed according to OECD (2004) guidelines. At 56 days of exposure, the juveniles were removed from the containers by placing them in a water bath at 60ºC, according to ISO (11268-2:1998). As the juveniles emerged at the soil surface, they were counted. All the endpoints (except for reproduction) were assessed before exposure (0d) on animals sampled directly from the acclimation container, containing LUFA 2.2. For the determination of metal and radionuclide body

burdens, as well as growth (measured after 14, 28 and 56 days of exposure) organisms were weighted (fresh weight) to the nearest 0.1 mg.

To ensure optimal conditions for earthworms' survival, weekly, approximately 5g of oatmeal was added to the containers; likewise deionised water was added, in order to maintain soil water content. After 28 days of exposure, the soils tested were renewed in all containers (except those from the reproduction assay), and only adult earthworms were moved to the new soils, in order to avoid competition with juveniles hatched during the 56 days experiment.

4.3.5 Radionuclide and metal body burdens

Following Maenpaa et al. (2002), the animals used for the assessment of metal body burdens were left to depurate for ±6 h in moistened filter paper and kept at -20ºC until analysis. For radionuclide body burdens analysis, the animals were left to depurate for 48 h in moist filter paper, weighted (fresh weight) and stored at -80ºC until analysis. In this case a longer depuration time was required to allow total clearance of the gut content. Existing standard toxicity protocols were not designed to assess body burden and consequently there are no internationally agreed standards for depuration time (Nahmani et al., 2007). Samples preparation and the analyses of radionuclides on organisms and soil samples were performed as described by Carvalho et al. (2007; 2009b).

The quantification of metal body burdens was performed with slight modifications of the methodology described by Antunes et al. (2008a). Hence, a pool of 4 earthworms from each replicate (5 replicates per exposure period and soil tested) was used and the digest was diluted to a final volume of 10 ml with Mili-Q water. Sample blanks were obtained following the same procedure, but without the biological sample. Inductively coupled plasma mass spectrometry (ICP/MS) (APHA, 1995) was used to determine the total concentrations of Be, Al, Ba, Mn, Fe, Ni, Zn, Sr, Se, Cd, Pb and U in whole body of earthworms exposed to LUFA 2.2 and to the contaminated soil. Results were expressed in $\mu g\ g^{-1}_{dw.}$

To determine metal bioaccumulation factor (BAF) in the earthworms, the ratio between the metal body burden in earthworms exposed to soil B ($\mu g\ g^{-1}$ dry weight) and total concentration in soil ($\mu g\ g^{-1}$ soil) was calculated for each metal, in all the exposure periods. For calculation purposes, the total metal concentrations found in soil B, by André and co-authors (2009), reporting a study carried out in parallel, were used.

Based on the study performed by Copplestone and co-authors (1999), concentration ratios (CRs) were calculated for radionuclides after 14 and 56 days of exposure. The concentration ratio is defined as the ratio between the activity concentration in earthworms (Bq Kg^{-1} dry weight) and the activity concentration in the soil (Bq Kg^{-1} soil).

4.3.6 Coelomocytes extrusion

Earthworm coelomocytes were obtained using the modified protocol of Reinecke et al. (2004). The same cell suspensions were used either for comet assay and flow cytometry analysis. Before use, the cell suspension was divided in two aliquots which were frozen at -80ºC in a cryopreservation medium, previously tested. For comet assay, cells were frozen in a solution containing 10% dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS). As for flow cytometry, the cryopreservation medium used was composed by 10% Glycerol, PBS and 10% fetal bovine serum (FBS).

4.3.7 Comet assay

Prior to alkaline comet assay, the cell suspension was placed at 37ºC to rapidly defrost, centrifuged 3 min at 380*g* and washed with PBS. The comet assay was conducted under yellow light, to prevent UV-induced DNA damage, and performed with slight modifications of the protocol described by Nogueira et al.(2006): briefly, microscope slides, were covered with the first agarose layer and left to dry; another layer containing the cells was placed on top of the first agarose layer.

Visual scoring of cellular DNA on each slide was based on the categorization of 100 randomly-selected cells. The comet-like formations were visually graded into five classes, depending on DNA damage and scored as described by García et al. (2004).

Positive controls were always included, and consisted of cells, previously exposed to 200 μM of H_2O_2, for 1 h.

4.3.8 Flow cytometry

Prior to flow cytometry analysis, cells were rapidly defrosted placed at 37ºC, centrifuged 3 min at 380*g* and washed with PBS.

After the identification and characterization of coelomocytes, the analyses of samples, were performed on a FACSCalibur™ (BD Biosciences, Erembodegem, Belgium). Cells were analysed on the basis of their size and complexity; eleocytes median fluorescence intensity (MFI); DNA content, as CV % of the G0/G1 DNA peak; proliferation, as S+G2+M cell frequency (%). For the last two analyses mentioned, cells were stained with propidium iodide using Cycloscope Reagent (Cytognos), according to the kit's protocol, and analysed using ModFit 2.0 software (Becton Dickinson Immunocytometry Systems). The remaining analyses were performed using Infinicyt 1.2 software (Cytognos). For size, complexity and MFI, 100.000 threshold events were analysed, per worm sample. Samples were then analysed in a FL1-H: FITC logaritmic/SSC-H plot. As for DNA analyses, 10.000 threshold events were collected, and samples were analysed on the basis of their FL2 fluorescence.

4.3.9 Statistical analysis

Bifactorial analyses of variance (ANOVAs) were used, to test the effect of exposure period and soil, on the parameters analysed. Dunnett's multi-comparison tests were used, when applicable, to discriminate significant differences between organisms exposed to a given period and the control group [organisms before exposure (0h)]. Student's t-tests were also used whenever a significant interaction between both factors was recorded, to compare organisms exposed to both soils for each exposure period. The level of significance defined for all the analyses was 0.05.

4.4 Results & Discussion

The analyses of our samples showed that soil B is highly contaminated with metals and radionuclides (please see Table 5 in chapter 3). Despite higher pH and organic matter values (please see Table 5 in chapter 3), earthworms exposed to soil B displayed significant higher concentrations of all the metals analyzed, when compared to those exposed to LUFA 2.2 soil (Table 6). The same was true for the radionuclides, as earthworms showed higher levels of all the isotopes herein analyzed, when compared to organisms exposed to LUFA 2.2 soil (Table 7). Metals and radionuclides were bioaccumulated by earthworms, probably through dermal contact with soil solution and ingestion of soil, contaminated food and soil water as suggested by Lanno and co workers (2004). Previous studies have reported that earthworms increase metals bioavailability, altering soil physical and chemical properties responsible for metal fractionation (Cheng and Wong, 2002; Udovic et al., 2007). Also, as observed by Weltje (1998), earthworms gut transit and

digestion can affect metals mobility due to its pH and favor their assimilation. After 14 days of exposure the levels of almost all metals remained relatively constant until the end of the exposure, suggesting that a homeostatic equilibrium was attained. However, the concentration of essential elements like Zn or Se continued to increase along time (Table 6) and the same happened to elements difficult to eliminate like Cd and U (Table 6). Regarding radionuclides, the levels of almost all the isotopes continued to increase up to 56 days of exposure in earthworms exposed to soil B, except for ^{230}Th, which levels decreased to less than half of the activity concentration recorded after 14 days of exposure (Table 7). The reasons behind the decrease of this isotope are not clear and require further studies. In order to predict doses to biota, it is essential to estimate radionuclide's transfer to biota to allow the quantification of internal dose (Beresford et al., 2004).

The calculation of bioaccumulation factors (BAFs) showed that barium was the element with the highest BAFs (Table 8), at all exposure periods. This may be explained by the characteristics of this soil, undergoing deposition of the sludge from the effluent treatment pond, as described in the Materials and Methods section. However, these considerations may not be straightforward, since this soil displays a complex mixture of contaminants, whose interactive effects are unknown. Cadmium presented BAF values indicative of low bioaccumulation (1<BAF<10) only at 14 and 56 days of exposure (Table 8), indicating that this element was slowly bioaccumulated throughout time. The concentration ratios (CRs) calculated for radionuclides (Table 9), were not significant for almost any of the radionuclides here analyzed. However, after 56 days of exposure it was possible to observe a CR of 1 for ^{226}Ra concordant with low (1<CR<10) bioaccumulation values (Table 9), probably indicating that radionuclides need more time to be bioaccumulated. The response of earthworms to metals exposure is reported to be species-specific (Cheng and Wong, 2002). For most species, metals and radionuclides are toxic, negatively affecting growth (Gestel et al., 1991; Khalil et al., 1996) and reproduction (Spurgeon and Hopkin, 1996; Alonzo et al., 2006). Herein, earthworm's exposure to soil B may have caused negative effects on both biological parameters. As shown in Fig. 17, earthworm's biomass decreased significantly when they were exposed to soil B, immediately after 14 days of exposure, which may reflect a decrease in the organism's fitness. The reproduction assay showed a complete inhibition of cocoon production, as no juveniles were detected, at the end of the experiment, in any of the 5 containers (replicates) with soil B. On the contrary, in the containers with LUFA 2.2, juveniles were detected in all 5 replicates. The number of juveniles in LUFA 2.2 soil was, in average, 110 organisms *per* replicate.

Table 6: Metal contents (µg g^{-1}dry weight) (mean ± standard deviation, *n* = 5) in *Eisenia andrei*. Letter (a) stand for statistical significant differences between unexposed (0 d) and exposed (1, 2, 7, 14, 56 days), after a Dunnet's multi-comparison test (p<0 05, following two-way ANOVA. Letter (b) stands for statistical significant differences between soils after a t-test, for each exposure period (p<0.05).

Soils	Time	Be	Al	Mn	Fe	Ni	Zn	Se	Sr	Cd	Ba	Pb	U
Earthworm metal content ± SD (µg.g^{-1} dry weight)													
Before exposure (0 d)		0.08 ± 0.02	998 ± 224.8	58.3 ± 11.8	866.2 ± 165	1.2 ± 0.17	91 ± 11	1.8 ± 0.5	8.6 ± 0.6	1.3 ± 0.1	14 ± 2.9	4.6 ± 0.8	0.37 ± 0.07
Lufa 2.2	1 d	0.13 ± 0.01$^{(a)}$	1504 ± 174.7$^{(a)}$	68.3 ± 11.6	1141 ± 85	1.5 ± 0.1$^{(a)}$	79 ± 4.5	1.8 ± 0.5	8 ± 0.6	1.2 ± 0.2	22 ± 2.8$^{(a)}$	5.8 ± 0.6	0.6 ± 0.2$^{(a)}$
	2 d	0.15 ± 0.04$^{(a)}$	1913 ± 411.6$^{(a)}$	78 ± 16.5$^{(a)}$	1331 ± 295	1.8 ± 0.3$^{(a)}$	75.5 ± 6.1$^{(a)}$	1.9 ± 0.8	8 ± 1	1.2 ± 0.2	25 ± 5.9$^{(a)}$	6.6 ± 1.7$^{(a)}$	0.5 ± 0.1
	7 d	0.16 ± 0.03$^{(a)}$	1547 ± 249.2$^{(a)}$	77 ± 9.9$^{(a)}$	1353 ± 251	1.9 ± 0.4$^{(a)}$	69.6 ± 4$^{(a)}$	1.7 ± 0.4	7 ± 0.6$^{(a)}$	1.5 ± 0.2	25 ± 3.2$^{(a)}$	6.8 ± 1$^{(a)}$	0.6 ± 0.05$^{(a)}$
	14 d	0.16 ± 0.04$^{(a)}$	1451 ± 302$^{(a)}$	72 ± 11.6$^{(a)}$	1326 ± 346.6	1.7 ± 0.2$^{(a)}$	70 ± 2.5$^{(a)}$	2.1 ± 0.3	6.7 ± 0.4$^{(a)}$	2.2 ± 0.2$^{(a)}$	22 ± 3.1$^{(a)}$	5.7 ± 0.9	1 ± 0.1$^{(a)}$
	56 d	0.13 ± 0.03$^{(a)}$	1250 ± 263	80 ± 22	1106 ± 246.6	1.4 ± 0.15	64.7 ± 7.8$^{(a)}$	2.7 ± 0.5	5.5 ± 0.5$^{(a)}$	3.2 ± 0.6$^{(a)}$	19 ± 4.6	5.1 ± 1.4	1 ± 0.3$^{(a)}$
Soil B	1 d	7.4 ± 2.8$^{(a,b)}$	4336 ± 1446.3$^{(a,b)}$	694 ± 245.6$^{(a,b)}$	2270 ± 723.3$^{(a,b)}$	14.5 ± 4.6$^{(a,b)}$	164 ± 24.8$^{(a,b)}$	2.5 ± 0.5	13 ± 2$^{(a,b)}$	1.5 ± 0.2$^{(b)}$	101 ± 39$^{(a.b)}$	2.5 ± 0.4$^{(a,b)}$	44 ± 15.5$^{(a,b)}$
	2 d	7.7 ± 2.6$^{(a,b)}$	4068 ± 1478.3$^{(a,b)}$	686 ± 229.7$^{(a,b)}$	2127 ± 725.9$^{(a,b)}$	14.6 ± 4.6$^{(a,b)}$	171 ± 27.3$^{(a,b)}$	2.4 ± 0.6	11 ± 2.4$^{(b)}$	1.7 ± 0.3$^{(a,b)}$	93 ± 34$^{(a.b)}$	1.8 ± 0.7$^{(a,b)}$	45 ± 15$^{(a,b)}$
	7 d	17.6 ± 4$^{(a,b)}$	9206 ± 2188.4$^{(a,b)}$	1579 ± 413.9$^{(a,b)}$	4786 ± 1157$^{(a,b)}$	32.7 ± 7.9$^{(a,b)}$	248 ± 36.8$^{(a,b)}$	2.5 ± 0.3$^{(b)}$	17 ± 3.3$^{(a,b)}$	2.3 ± 0.3$^{(a,b)}$	219 ± 46.9$^{(a,b)}$	3.1 ± 0.7$^{(b)}$	100 ± 25$^{(a,b)}$
	14 d	15.1 ± 2.2$^{(a,b)}$	7996 ± 1281$^{(a,b)}$	1390 ± 210$^{(a,b)}$	4045 ± 701$^{(a,b)}$	28.7 ± 3.5$^{(a,b)}$	233 ± 22.5$^{(a,b)}$	2.6 ± 0.2$^{(a,b)}$	16 ± 2.6$^{(a,b)}$	2.6 ± 0.4$^{(a)}$	191 ± 36.5$^{(a,b)}$	3.1 ± 1.2$^{(b)}$	86 ± 12$^{(a,b)}$
	56 d	14.6 ± 2.1$^{(a,b)}$	7476 ± 1232$^{(a,b)}$	1246 ± 245$^{(a,b)}$	3708 ± 846.5$^{(a,b)}$	26.4 ± 4.4$^{(a,b)}$	251 ± 6$^{(a,b)}$	5.1 ± 0.5$^{(a,b)}$	14 ± 2$^{(a,b)}$	4.3 ± 0.7$^{(a)}$	162 ± 32$^{(a,b)}$	2.6 ± 0.4$^{(a,b)}$	91± 16$^{(a,b)}$

Table 7: Radionuclide concentrations (Bq Kg^{-1} dry weight) (mean ± standard deviation) in *Eisenia andrei*, before exposure (0), after 14 days (14) and 56 days of exposure (56).

Soils	Time	^{238}U	^{235}U	^{234}U	^{230}Th	^{226}Ra	^{210} Pb	^{232}Th
Earthworm radionuclides concentration ± SE (Bq Kg^{-1} dry weight)								
Before exposure (0d)		2.8 ± 0.1	0.2 ± 0.04	3.1 ± 0.4	5.7 ± 0.7	52.9 ± 0.2	5.23 ± 0.27	2.8 ± 0.4
Lufa 2.2	14 d	6.2 ± 0	0.3 ± 0.03	7.7 ± 0.2	0.9 ± 0.1	565 ± 37.5	8.3 ± 4.7	0.7 ± 0.2
	56 d	11.5 ± 2.6	0.4 ± 0.06	12.3 ± 2.4	1.2 ± 0.5	778.1 ± 20.2	4.8 ± 0.5	1.5 ± 1
Soil B	14 d	157.6 ± 22.3	7.1 ± 1.2	155 ± 21.1	153.6 ± 32.5	429.3 ± 170.7	54.3 ± 16.6	0.6 ± 0.2
	56 d	304.5 ± 14.2	13.2 ± 0.6	297 ± 13.1	60.1 ± 21.3	1540 ± 417.2	66.6 ± 2.2	12.1 ± 8.9

Table 8: Bioacumulation factors (BAF) in *Eisenia andrei*, after 1, 2, 7, 14 and 56 days of exposure to the contaminated soil from the Cunha Baixa uranium mine (soil B).

Time	Be	Al	Mn	Fe	Ni	Zn	Sr	Cd	Ba	Pb	U
1 day	0.15	0.16	0.19	0.17	0.16	0.43	0.8	0.6	11.9(**)	0.26	0.21
2 days	0.15	0.15	0.18	0.16	0.16	0.42	0.67	0.66	10.9(**)	0.18	0.21
7 days	0.35	0.35	0.42	0.36	0.36	0.51	0.74	0.88	25.7(**)	0.32	0.46
14 days	0.30	0.30	0.37	0.30	0.31	0.49	0.67	1(*)	22.5(**)	0.32	0.40
56 days	0.29	0.28	0.34	0.28	0.29	0.57	0.66	1.66(*)	19.15(**)	0.26	0.42

Symbols (*) represent BAF values concordant with low (1<BAF<10) bioaccumulation values and (**) represent values concordant with intermediate (10<BAF<100) bioaccumulation values.

Table 9: Radionuclides concentration ratios (CRs) for Eisenia andrei, after 14 and 56 days of exposure to the contaminated soil from the Cunha Baixa uranium mine (soil B).

Time	^{238}U	^{235}U	^{234}U	^{230}Th	^{226}Ra	^{210}Pb	^{232}Th
14 days	0.04	0.04	0.04	0.01	0.29	0.03	0.02
56 days	0.08	0.08	0.08	0.01	1.02(*)	0.50	0.03

Symbols (*) represent CR values concordant with low (1<CR<10) bioaccumulation values and (**) represent values concordant with intermediate (10<CR<100) bioaccumulation values.

All the validity criteria for this test were fulfilled according to the ISO (1996) guideline: no mortality of adults was recorded for the organisms exposed to the control soil LUFA 2.2, the rate of production of juveniles per control container was ≥30 % and the CV of juveniles in the control was ≤30 % (26%). These results showed that soil B, a soil highly contaminated with metals and radionuclides, had seriously compromised parameters (growth and reproduction) that are crucial for the sustainability of earthworm's population.

Comet assay was used to asses DNA damages, measuring DNA integrity in coelomocytes non-invasively retrieved from earthworms. Comet assay, is a sensitive tool for the identification and quantification of genotoxicity (Martin et al., 1999; Faust et al., 2004; Qiao et al., 2007), that allows the detection of low levels of DNA damage in single cells (Tice et al., 2000). Undoubtedly, the most important damages at the molecular level are the induction of various types of lesions in the DNA, like, for example, strand breaks, which are effectively measured by comet assay (Jha, 2008). The level of strand breakage in DNA has been proposed as a sensitive indicator of genotoxicity and an effective biomarker in environmental biomonitoring (Shugart and Theodorakis, 1998; Shugart, 2000). The study of coelomocytes in earthworms is relevant since these immunocompetent cells, are particularly exposed to soil pollutants and are involved in the process of cell immunity (Dhainaut and Scaps, 2001; Manerikar et al., 2008). The results showed that the integrity of coelomocyte's DNA was significantly affected in earthworms exposed to soil B (Fig. 18), since damages were always significantly higher than those observed in organisms exposed to LUFA 2.2 (Fig. 18). The loss of DNA integrity may determine changes in the DNA molecule, including the induction of mutations and chomossomal aberrations (Hartwig and Schwerdtle, 2002; Jha, 2008). These damages may be caused by the exposure to the contaminated soil, since it contains metals and radionuclides that are major DNA damaging agents. Some metals induce the production and intracellular accumulation of reactive oxygen species (ROS), capable of yielding DNA damages (Ercal et al., 2001; Valko et al., 2005). Enhanced generation of ROS can overwhelm cell's intrinsic antioxidant defenses, and result in a condition known as "oxidative stress" (Ercal et al., 2001). Cells under oxidative stress display various dysfunctions due to lesions caused by ROS to lipids, proteins and DNA (Ercal et al., 2001). Ionizing radiation emitted by the radionuclides present in the soil, may also induce DNA damages. In this study, we have analyzed some radionuclides present in the ^{238}U radioactive series, namely, ^{235}U, ^{230}Th, ^{210}Pb and ^{226}Ra. ^{226}Ra is a radionuclide with major environmental implications, because it is on the top of an important sub-chain in which ^{222}Rn and ^{210}Pb are included, and perhaps the main contributors to the total dose for humans (UNSCEAR, 2000). Almost all of the radionuclides herein

analyzed, are mainly alpha-emitters, thus the ingestion of these elements represent serious risks to all organisms exposed to them (Carvalho and Oliveira, 2007). Alpha particles (high energy, relatively large mass and momentum, low velocity) have relatively high linear energy transfer (LET) values (Harrison and Day, 2008). Clustered DNA damage and double strand breaks (DSB), together with the degree of complexity of the damage, have been shown to increase with LET (Valentin, 2003; Costes et al., 2010). The ultimate biological consequence is dependent on whether the damage can be repaired and with what fidelity (Harrison and Day, 2008). Types of clustered damage may compromise DNA repair fidelity and can lead to an increase in mutation frequency (Pearson et al., 2004). DNA double strand breaks (DSB) are the most detrimental type of molecular damage. It is assumed that DSB cause toxic effects if not repaired or mutations if repaired incorrectly (Belyaev 2010). It is thought that unrepaired DSB may lead to death and misrepaired DSB may lead to viable chromosomal rearrangements (Costes et al., 2010). Some of these rearrangements may be instrumental in the development of cancer (Costes et al., 2010). In the organisms exposed to LUFA 2.2 soil it was observed that DNA damage increased with time, since significant differences for all the exposure periods were observed when compared with 0 h of exposure (Fig. 18). This damage may be due to the exposure to metals such as Pb, Al and Sr present in LUFA 2.2 soil. However, these values were always significantly lower than those observed in the organisms exposed to the contaminated soil.

Flow cytometry was used to evaluate potential cytotoxic and also genotoxic effects caused by the exposure of earthworms to the soil B. To that end, parameters such as cell frequency, cell DNA content, median fluorescence intensity (MFI) and cell proliferation were evaluated in coelomocytes from exposed animals (Fig. 17 and 18). Results showed that the immune system of these organisms was affected, since the frequency of the different types of coelomocytes, was altered in the earthworms exposed to soil B (Fig. 18). Also, in these organisms exposed to soil B, the eleocytes frequency increased significantly since the second day and until the end of the exposure (Fig. 18). This may be due to the fact that these cells are responsible for the maintenance of the proper functioning of the whole organism, as they maintain constant pH and ionic balance of both coelomic fluid and hemolymph (Affar et al., 1998; Adamowicz, 2005). Moreover, these cells have also the capacity to store endogenous and exogenous materials like metals (Affar et al., 1998). Therefore, the increase in eleocytes frequency may be an attempt of the organisms to survive by adapting to the surrounding environment. The frequency of amebocytes and granulocytes was also affected, and displayed a significant decrease immediately after the second day of exposure until the end of the experiment (Fig. 18).

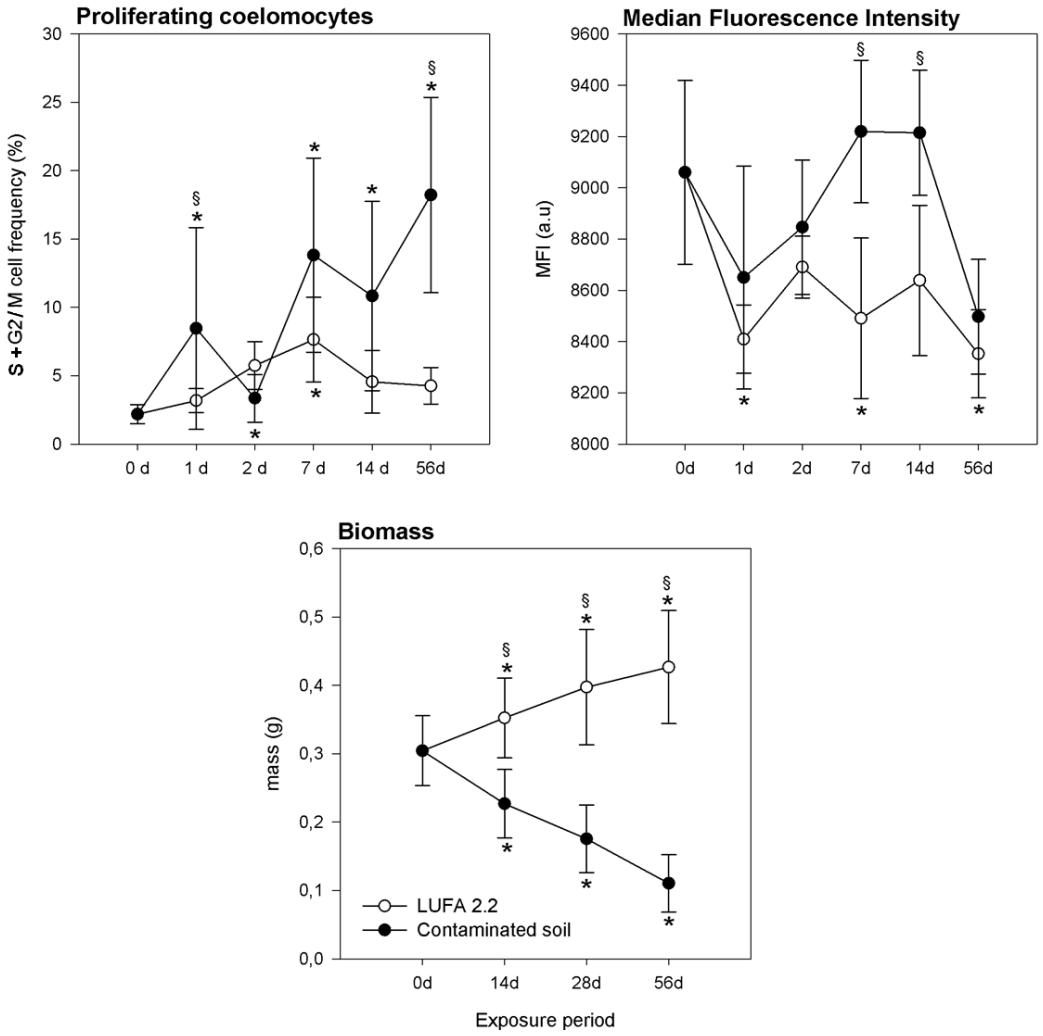

Figure 17: Coelomocytes proliferation, eleocytes median fluorescence intensity (MFI) and biomass of earthworms exposed to a contaminated soil (black circles) and to LUFA 2.2 (white circles). Parameters were assessed before (0 d) and after 1, 2, 7, 14 and 56 days of exposure for all analyses. Data are shown as average ± standard error. (*) stands for statistical significant differences after a Dunnet's multi-comparison test ($p<0.05$), to compare exposure periods, of each soil tested, with 0 d. (§) stands for statistical significant differences after a Student's t-test ($p<0.05$), to compare soils at each exposure period

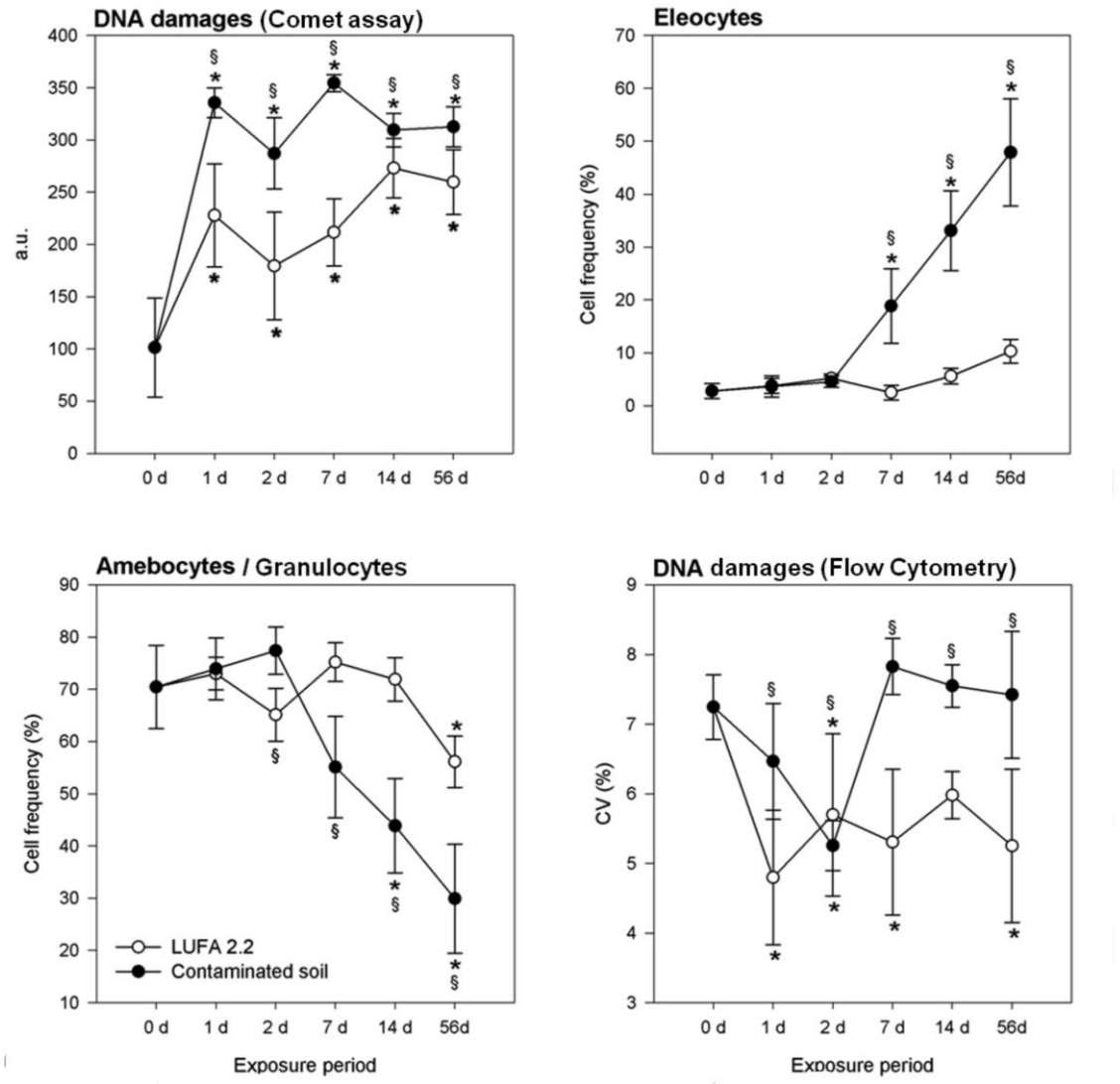

Figure 18: Coelomocytes DNA integrity (assessed by comet assay and flow cytometry), eleocytes and amebocytes/granulocytes frequency in earthworms exposed to a contaminated soil (black circles) and to LUFA 2.2 (white circles). Parameters were assessed before (0 d) and after 1, 2, 7, 14 and 56 days of exposure for all the analyses. Data are shown as average ± standard error. (*) stands for statistical significant differences after a Dunnet's multi-comparison test ($p<0.05$), to compare exposure periods, of each soil tested, with 0 d. (§) stands for statistical significant differences after a Student's t-test ($p<0.05$), to compare soils at each exposure period.

These cells have effector functions like phagocytosis, encapsulation, nodulation and humoral immune responses (Adamowicz, 2005), which means that depletion in these cells may render earthworms more vulnerable to external factors. A decrease in the number of these cells may have been caused by the exposure to metals, since they are able to adversely affect the immune system of wildlife species, including invertebrates (Galloway and Depledge, 2001), compromising immune cells viability and effector functions (Galloway and Depledge, 2001; Homa et al., 2003).

Radionuclides are able to interfere with the immune response of a variety of animal species (including humans) at exposure levels below which, other toxicity endpoints do not respond (Giulio and Hinton, 2008). For instance, the radionuclides of uranium and thorium decay series can be toxic chemicals for the immune system (Sheppard et al., 2005; UNSCEAR, 2006). High doses of radiation produce immune suppression mainly due to the destruction of cells. At low doses and dose rates, the effects may be suppressive or stimulatory (UNSCEAR, 2006). In this study it is possible to observe not only suppression, but also stimulation of earthworm's immune system. As shown in Fig. 17, there is an increase in coelomocytes proliferation in earthworms exposed to soil B and significant differences between organisms exposed to both soils were observed after 56 days of exposure. This may be related to the increase in eleocytes frequency. Median fluorescence intensity (MFI) was determined in earthworm's eleocytes. Eleocytes are easily detectable by flow cytometry due to the presence of granules (chloragosomes), and characteristic autofluorescence derived from riboflavin and other fluorophores like lipofuscins, stored in chloragosomes (Cholewa et al., 2006; Koziol et al., 2006). The autofluorescence of these cells decreased in the first 24 h of exposure and then increased, in earthworms exposed to soil B (Fig. 17). The levels were somewhat constant from 7 to 14 days of exposure and after that period started to decrease (Fig. 17). This reveals a bell shape trend, similar to what was observed by Gastaldi and co-authors (2007). Due to high variability between samples, significant differences between both soils were only observed at day 7 and 14 of exposure (Fig. 17). The decrease observed in the first 24 h in both soils, may be due to the decrease of riboflavin in eleocytes chloragosomes, caused by the accumulation of metals in earthworms of both soils, when comparing with 0 h of exposure (Fig. 17). Similar results were observed by Plytycz and co-authors (2009) that suggest riboflavin as good marker of soil metal pollution, because of its decrease in the coelomocytes of field worms inhabiting three metalliferous soils. However, in our study after 2 days of exposure there was an increase in eleocytes autofluorescence, in earthworms exposed to soil B (Fig. 17). This may be caused by the presence of another fluorophore, the lipofuscin. There are evidences, that lipofuscin may be the second fluorophore responsible for eleocytes autofluorescence (Koziol et al., 2006; Homa et al., 2010). Reactive oxygen species (ROS) produced in both physiological and stress conditions can stimulate the peroxidation of membrane lipids, whose end-products accumulate within the lysosomes as insoluble pigments known as lipofuscins (Brunk and Collins, 1981). Gastaldi and co-authors (2007), observed that the exposure to metals can stimulate lipofuscinogenesis in the chloragogeneous tissue (the precursor cells of eleocytes). The decline of autofluorescence after 56 days of exposure (Fig. 17) , a phenomenon also observed

by Gastaldi and co-authors (Gastaldi et al., 2007), could be partly due to exocytosis of residual bodies containing lipofuscin during longer times of exposure, or to eleocytes extrusion through dorsal pores and excretory system (Honeycutt et al., 1995; Sample et al., 1999). Residual body exocytose may explain the decrease in eleocytes autofluorescence, despite the increase in eleocytes number, observed (Fig 17 and 18). In the present study, flow cytometry was also used to assess genotoxicity. Cell's DNA content was evaluated, in order to assess the amount of chromosomal damage, as it is reflected by an increase in cell-to-cell variation in DNA content (Matson et al., 2004). The exposure of earthworm's to the contaminated soil has caused significantly higher DNA damages, as shown in Fig. 18, when compared to those observed when earthworms were exposed to LUFA 2.2. However these were not significantly different from those observed at time 0 h. The level of DNA damages detected at this sampling period was not clear and need further studies. As shown in Fig. 18, DNA damages decreased until the second day of exposure and then increased after 7 days till the end of the experiment. These results were consistent with those detected by comet assay, as can be seen by the analysis of the two plots. However, after 1 day of exposure, DNA damages were detected by comet assay and not by flow cytometry, which is probably related to the fact that comet assay is a more sensitive technique, since it detects damages at an earlier stage. Comet assay detects strand breaks, which can lead to chromosomal damages that may subsequently be detected by flow cytometry.

The effects observed in the organisms exposed to soil B result from the combined effects of both radiation and chemical exposure, since the experimental design did not allow the distinction between both factors. This other aspect will be addressed in future experiments.

A trial experiment was performed under laboratory conditions (Lourenço et al., 2011), where the DNA damages recorded by comet assay were somewhat lower; however the differences between the laboratory and the *in situ* trial were not significant. The eleocytes and amoebocytes frequency recorded in the laboratory experiment were highly different from the *in situ* experiment. The reasons of such differences are yet to be clarified. Moreover, concentrations of metals and radionuclides are higher in earthworms exposed *in situ* than in the ones from the laboratory exposure, probably due to the natural fluctuating environmental conditions, that may potentiate the intake of these contaminants.

4.5 Conclusion

This study showed that the evaluation of genotoxicity and cytotoxicity endpoints, along with other parameters at individual level in standard reproduction assays conducted *in situ,* may reduce the underestimation of risks in the evaluation of areas contaminated with uranium and other radioactive mining wastes. Comet assay and flow cytometry proved to be useful techniques to be coupled with *in situ* assays, since they allowed the early detection of important sublethal responses in earthworms exposed to metals and radionuclides, which can later affect the overall fitness of these organisms, including their reproductive success. All the sublethal responses evaluated in earthworms exposed to metals and radionuclides, namely, growth, reproduction, the bioaccumulation of metals and radionuclides, DNA damages, cell-to-cell variation in DNA content, MFI, coelomocytes frequency and coelomocytes proliferation, showed a positive response in exposed organisms.

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CHAPTER 5 - SSH GENE EXPRESSION PROFILE OF *EISENIA ANDREI* EXPOSED IN SITU TO A NATURALLY CONTAMINATED SOIL FROM AN ABANDONED URANIUM MINE

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5.1 Abstract

The effects of the exposure of earthworms (*Eisenia andrei*) to contaminated soil from an abandoned uranium mine, were assessed through gene expression profile evaluation by Suppression Subtractive Hybridization (SSH). Organisms were exposed *in situ* for 56 days, in containers placed both in a contaminated and in a non contaminated site (reference). Organisms were sampled after 14 and 56 days of exposure. Results showed that the main physiological functions affected by the exposure to metals and radionuclides were: metabolism, oxireductase activity, redox homeostasis and response to chemical stimulus and stress. The relative expression of *NADH dehydrogenase subunit 1* and *elongation factor 1 alpha* was also affected, since the genes encoding these enzymes were significantly up and down-regulated, after 14 and 56 days of exposure, respectively. Also, an EST with homology for *SET* oncogene was found to be up-regulated. To the best of our knowledge, this is the first time that this gene was identified in earthworms and thus, further studies are required, to clarify its involvement in the toxicity of metals and radionuclides. Considering the results herein presented, gene expression profiling proved to be a very useful tool to detect earthworms underlying responses to metals and radionuclides exposure, pointing out for the detection and development of potential biomarkers.

Keywords: Earthworms; metals; radionuclides; gene expression; biomarker

5.2 Introduction

The emergence of molecular biology techniques applied to ecotoxicology, allowed a better understanding of the impact of contaminants in living organisms (Brulle et al., 2008). Responses to environmental stressors, including potentially toxic chemical compounds, are inevitably accompanied by changes in gene expression profiles in receptor organisms (Brulle et al., 2010). Indeed, gene expression profiles represent the first level of integration between environmental stressors and the genome, which, through the synthesis of proteins, pilots the response of the organisms to external changes (Brulle et al., 2008; Wang et al., 2010a). Thus, the analysis of changes in gene expression is a powerful tool to diagnose the existence of a stress and to analyze the response to a stressor (Brulle et al., 2008; Bernard et al., 2010). Some molecular-genetic tools, widely developed in the last decade, allow the analysis of transcriptomes of stressed organisms, exposed in laboratory or under field conditions (Lee et al., 2005; Pirooznia et al., 2007; Brulle et al., 2010). These tools include cDNA microarrays, real-time quantitative PCR (RT-qPCR), pyro-sequencing technologies, expressed sequence tags (ESTs) sequencing and Suppression Subtractive Hybridization PCR (SSH), among others. Consequently, it is now possible to identify the pattern of differential gene expression (which genes are up or down-regulated), in organisms under defined exposure conditions and defined life-cycle phases (Brulle et al., 2010).

Suppression Subtractive Hybridization PCR (SSH), a technique developed by Diatchenko and collaborators (1996), is an effective method for the generation of subtracted cDNA libraries. It is based primarily on a technique designated by suppression PCR and combines normalization and subtraction in a single procedure. The normalization step equalizes the abundance of cDNA within the target population and the subtraction step excludes the common sequences between the target and the control populations (Diatchenko et al., 1996). In a model system, the SSH technique can enrich for rare sequences over 1.000-fold in one round of subtractive hybridization. After the SSH, the cDNA fragments may be used as probes on microarrays or cloned to generate expressed sequence tags (ESTs). In the absence of full genome sequences, ESTs allow rapid identification of expressed genes by sequence analysis and are an important resource for comparative and functional genomic studies and to the discovery of new genes during specific developmental and physiological events (Lee et al., 2005; Pirooznia et al., 2007). ESTs are often generated from randomly selected cDNA clones and provide valuable transcriptional data for the annotation of genomic sequences (Pirooznia 2007).

Earthworms are widely used as model organisms in terrestrial ecotoxicology, and are increasingly recognized as sentinel species (Pirooznia et al., 2007; Wang et al., 2010a). These

organisms are very important for the preservation of the ecosystem function, by playing a pivotal role in increasing soil fertility and turnover (Lee et al., 2005). Due to their ecological importance, they have been used as test species for the risk assessment of chemical substances, in standard protocols proposed by international organizations such as the Organization for Economic Cooperation and Development (OECD) and the International Organization for Standardization (ISO) (Yoshida et al., 2009). Such protocols were designed to assess effects on individual endpoints (e.g. survival and reproduction output). However, biota living in close contact with contaminated soils may experience stress at different levels of biological organization, from the molecular-genetic to ecological and community levels (Brulle et al., 2010). Thus, the detection of molecular changes that occur in response to a pollutant exposure, in these organisms will not only help in the assessment of current biomonitoring procedures, but will allow for the identification of potential diagnostic and prognostic markers (Stürzenbaum et al., 1998).

In the present study, Suppression Subtractive Hybridization PCR (SSH) was employed to assess the gene expression profile of earthworms exposed *in situ,* to a natural soil from an abandoned uranium mine area, contaminated with metals and radionuclides. The aim was to identify the main biological pathways and cellular physiological functions that were affected by the exposure to the contaminants. Additionally, we expected to identify potential new genetic biomarkers of earthworm's exposure to metals and radionuclides that could be used in future studies.

5.3 Materials and Methods

5.3.1 Study site

The Cunha Baixa uranium mine (coordinates: 40.571393 N 7.753322 W), is one of the largest uranium mines in Portugal (Carvalho et al., 2009). The mine is located approximately 20 km southeast of Viseu, near the Cunha Baixa village. The underground and open pit mining works, carried out between 1970–1993, produced about 1000 t of U_3O_8 and one million tonnes of waste materials which were disposed of in a dump surrounding the mine area (Neves et al., 2005). Sulfuric acid was used for *in situ* leaching of uranium in underground works as well as in heap leaching of low grade ores at the surface (Antunes et al., 2008a; Pereira et al., 2008; Carvalho et al., 2009). The mine pit water is, therefore, acidic (pH <3.5) and is able to move through permeable heap leaching wastes, following changes in the piezometric level of the underlying aquifer, mobilizing elements and increasing their dispersion (Neves et al., 2005). Thus, the open

pit area presents a risk to surface and groundwater resources being a source of metals and radionuclides (Neves et al., 2005). Since the end of the mining activity, the acidic water from the mine pit has been almost continuously pumped out to treatment plants and treated with burned lime and barium chloride for pH neutralization and radionuclides precipitation, respectively (Pereira et al., 2008; Carvalho et al., 2009). When the maximum capacity of the pond is reached, the sludge formed by the accumulation of the chemical compounds at the bottom is removed and spread near the mine pit. The sludge has high levels of metals (Pereira et al., 2008) and radionuclides from uranium series and low levels of radionuclides from the thorium series (Carvalho et al., 2007). Soils from the adjacent areas of the mine pit have suffered several impacts, through the deposition of both tailings and sludge from the waste water treatment plant (the latter still ongoing) (Antunes et al., 2008a; Pereira et al., 2008).

5.3.2 Soils tested

Previous studies evaluated the degree of contamination and toxicity profiles of several soils adjacent to the Cunha Baixa uranium mine (Antunes et al., 2008b; Pereira et al., 2008). Based on that information, two sites were selected for the current study: i) site B, used for the deposition of sludge from the waste water treatment plant, with a radiation level of 850 cps and highly contaminated with metals and radionuclides and ii) site I, selected as the reference site due to its low radiation level (414 cps) and distance from the mine pit (Antunes et al., 2008b; Pereira et al., 2008). The soil from site B (soil B), was chosen as the test soil, since it displayed high extractable concentrations of metals, namely U, Mn, Al and Sr (Pereira et al., 2008) and radionuclides (Lourenço et al., 2012). Additionally, soil B has shown to affect growth, reproduction, DNA (genotoxicity), immune cells (cytotoxicity) and tissues in earthworms (Lourenço et al., 2011a; Lourenço et al., 2012) and has also presented high toxicity for plants (Pereira et al., 2009). The standard reference soil LUFA 2.2 (Speyer, Germany) was used as control soil.

After removing the superficial layer (plant debris), the first 20 cm of soil were collected and sieved to discard the >2 mm fraction. Water holding capacity (WHC), water content (%) and pH of both soils were measured and soil water content was adjusted to 40% of WHC_{max}. Water holding capacity (WHC) of soils was determined as described by ISO protocols (ISO, 11268-2:1998, 17512-1:2008). Soil water content was determined considering the weight loss after drying the soils at 105 °C, for 24 h. Soil pH was measured in a soil–water suspension (1:5 w/v extraction ratio) according to the method described in FAOUN (1984).

5.3.3 Test organisms

The test organisms (*Eisenia andrei*) were obtained from synchronised laboratorial cultures, reared in large containers, under controlled temperature conditions (20±2 °C) and a photoperiod of 16 h^L: 8 h^D. The substrate used for the cultures, composed by sphagnum peat, horse manure and $CaCO_3$ (to adjust the pH between 6 and 7), was periodically moistened with distilled water and the pH monitored each week. Earthworms were fed twice a month with dry and defaunated manure.

For this study, adult earthworms with clitellum and body mass between 250 and 600 mg were used, as recommended by international guidelines (OECD, 2004; ISO, 11268-2:1998). Before exposure, the organisms were acclimated for 24 h in containers with the soil LUFA 2.2.

5.3.4 *In situ* exposure

Organisms were exposed in 1-L plastic buckets (12 cm height and 10 cm in diameter), with lids bearing two openings, one at the top and another at the bottom, for ventilation (Antunes et al., 2008a). The openings were covered with 300 μm nylon mesh, using white thermal glue (supplied by Elis–Taiwan, Taiwan, ref. TN122/WS), which has been shown to be non-toxic to aquatic invertebrates (Pereira et al., 2000). The containers that were placed in site B were filled with 500g of soil B, and those placed in site I, were filled with 500g of LUFA 2.2, to eliminate both the impact of chemical contamination and radiation. The *in situ* assay began with the introduction of 10 earthworms in each container; the containers were then placed inside a depression made in the soil of each exposure site. The temperature of the soil at each exposure site was, at noon, 17.2ºC and 16.3ºC in sites I and B, respectively. The temperatures were measured using a Dostmann electronic_ GmbH P400 Series (TPCAL 100/25 DkD, Prema 3040) thermometer.

Twelve containers were used for each soil tested, at each exposure site. Six were sampled after 14 days of exposure and the remaining 6 were sampled at the end of the exposure (day 56). At the end of each exposure period, earthworms were removed from the chambers of each soil (6 chambers with a total of 60 animals). RNA was extracted from 5 animals from each experimental condition and the remaining animals were used to measure other parameters, namely metals and radionuclides bioaccumulation, DNA damages and analysis of immune cells by flow cytometry (data not shown).

To ensure earthworms' survival along the experiment, weekly, approximately, 5g of oatmeal was added to the containers; likewise deionised water was added, in order to maintain soil water

content. After 28 days of exposure, the soils tested were renewed in all containers, and only adult earthworms were moved to the new soils. The exposure periods were chosen according to the standard earthworm reproduction test (OECD, 2004), with the purpose of reflecting the acclimated physiological state rather than the initial stress phase.

5.3.5 Suppression subtractive hybridization (SSH)

Differentially expressed genes of earthworms exposed to soil B and to the reference soil LUFA 2.2, were identified by SSH, after 14 and 56 days of exposure. Five earthworms were used for each of the 4 groups (LUFA 2.2 and soil B, after 14 and 56 days of exposure). Earthworms were fixed in RNA later (Qiagen, Germany) and stored at -80ºC. Total RNA was extracted from earthworm's whole body using PureLink™ RNA Mini Kit (Invitrogen, USA) and treated on column with PureLink™ DNase (Invitrogen, USA) and quantified using Qubit™ Fluorometer (Invitrogen, USA) and Quant-iT™ RNA Assay Kit (Invitrogen, USA). The extracted RNAs were also checked for quality by visually assessing the ratio of *28S*:*18S* RNA on agarose gel stained with EtBr. Since the concentration of the RNA extracted from individual earthworms was not sufficient to meet the concentration required to perform SSH, for each group, equal amounts of RNA extracted from each earthworm were pooled. cDNA was synthesized from 1μg of total RNA, using SMARTer™ Pico PCR cDNA Synthesis Kit (Clontech, France). The cDNA generated was directly used for SSH. The forward- and reverse-subtracted libraries were generated using PCR-Select™ cDNA Subtraction Kit (Clontech, France), according to the manufacturer's protocol. The differential PCR products generated were cloned using The Original TA Cloning® Kit, with pCR® 2.1 vector (Invitrogen, USA) and *E. coli* JM109 competent cells (Promega, USA). A total of 130 randomly selected colonies were sequenced (Stabvida, Portugal) directly from the PCR product. Sequences were submitted to database searches using NCBI softwares Blastx and Blastn. When Blastx and Blastn searches were negative, conserved domain searches were performed using the NCBI Conserved Domain Database (CD-search), which uses RPS-BLAST. Gene function was inferred from sequences identified and reported in the Genbank database and in the ''Gene Ontology'' classification (http://www.geneontology.org/). Sequences similarities were considered to be significant when the expected values (E-values) were less than 10^{-3}.

The differential screening was confirmed by qPCR, using 5 genes: chitinase*, cytochrome c oxidase subunit III, putative elongation factor 1 alpha, NADH dehydrogenase subunit 1 and SET translocation (myeloid leukemia-associated) B*. The genes were chosen according to their

functions and their supposed response to this kind of contamination. The analysis of the expression levels was performed using 5 earthworms exposed to soil B and 5 earthworms exposed to LUFA 2.2 soil. The quantitative measurements were normalized using *Eisenia andrei 28S* rRNA as the reference gene (Procházková et al., 2011). First-strand cDNA was synthesized using SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen, USA). First-strand cDNA was synthesized from 1μg of total RNA in the presence of random hexamer primers. qPCR reactions were performed in a final volume of 20 μl containing 10 μl of SsoFast™ EvaGreen® Supermix (Biorad, USA), 1μl of diluted (1:10) cDNA and 200nM primers. A non-template control (NTC) was included in the qPCR analysis to determine the specificity of target cDNA amplification and also a non-RT control (NRT), including RNA instead of cDNA, to determine if the RNAs were contaminated with genomic DNA. The RT-qPCR was performed using the CFX96™ Real-Time System (Biorad, USA) and the following cycling parameters: 98ºC for 30 s and 44 cycles of 5 s at 95ºC and 10 s at 57ºC. Melting curves were performed to identify the presence of primer dimers and to analyze the specificity of the reaction. The amplification efficiency of each primer pair was calculated using a dilution series of cDNA. The primers used in RT-qPCR are listed in Table 10. The relative expression levels of target genes were calculated according to the efficiency corrected method described by Pfaffl (2001).

5.3.6 Statistical analysis

The statistical analysis, of the data obtained by real-time qPCR, was performed using the methodology described by Willems et al. (2008), that is based on a series of sequential corrections that can be used in highly variable data sets, to draw statistically reliable conclusions. After the application of this method, T-tests were performed to test for differences between control and contaminated groups. The level of significance defined for all the analyses was 0.05.

Table 10: Sequences of the primers used in RT-qPCR, for the determination of the relative expression levels of the genes used in the differential screening confirmation.

Gene	Sequence (5'- 3')
28S	For: CCTCAGTAACGGCGAGTGAA
	Rev: AGCGAACAAGTACCGTGAGGG
Chitinase	For: TACTGGCCCTTGAAATCGTC
	Rev: TTTGCTCCCAGATCAGAAGG
Elongation factor 1A	For: TCTTGACATTGAAGCCAACG
	Rev: TGCCCCTTCAGGATGTCTAC
NADH dehydrogenase subunit 1	For: GGTTGCCCAAACAATCTCAT
	Rev: TGTTTCTGCGAGGTTTGTGA
cytochrome c oxidase subunit III	For: AAACACCGCAGTCCTACTCG
	Rev: GGCCCCTAAGCATACTGTGA
SET translocation (myeloid leukemia-associated) B	For: CTGGGTTACTGCGTTTGTCA
	Rev: TCAAATTCCTGGACCTCGAC

5.4 Results and Discussion

The sequences of some of the clones retrieved from the analyzed libraries, at each exposure period, mainly matched to genes from earthworms, snails and nematodes (Table 12 and 13). The remaining sequenced clones showed homology to unidentified hypothetical or novel proteins or showed no homology with the sequences deposited in the database. It is also important to refer that only some of the clones obtained for each library, were sequenced, which means that the genes reported in the present work represent a fraction of the totality of genes that had their expression levels altered.

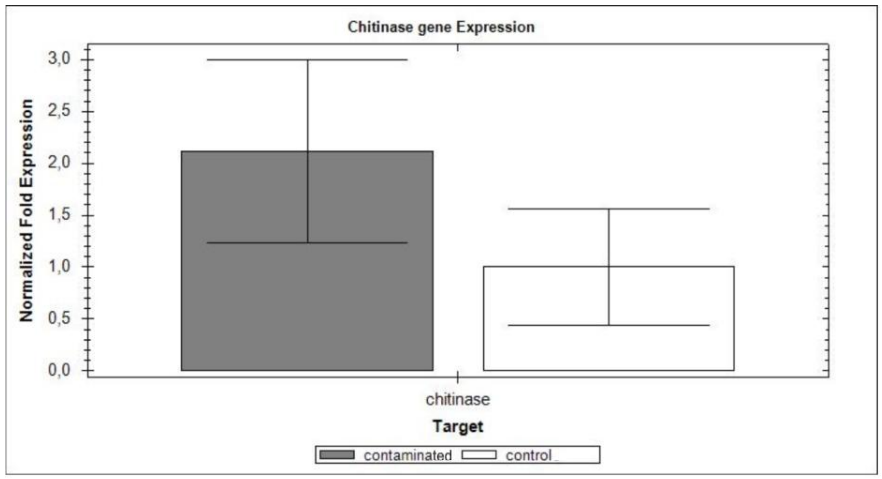
One hundred and thirty ESTs were associated to sixteen major cellular physiological functions namely, cell motility; muscle proteins; cell signaling; cytoskeletal proteins; homeostasis; calcium homeostasis; immunology; cell differentiation; redox homeostasis; metabolism; translation; nucleic acid binding; response to stress; oxireductase activity; nucleosome assembly; transporter activity (see Table 11 for library and library details). Only the most relevant in the context of the present study were discussed in more detail. The results showed that the main biological pathways and cellular physiological functions, affected by the exposure to contaminants were: metabolism, oxireductase activity, redox homeostasis and response to chemical stimulus and

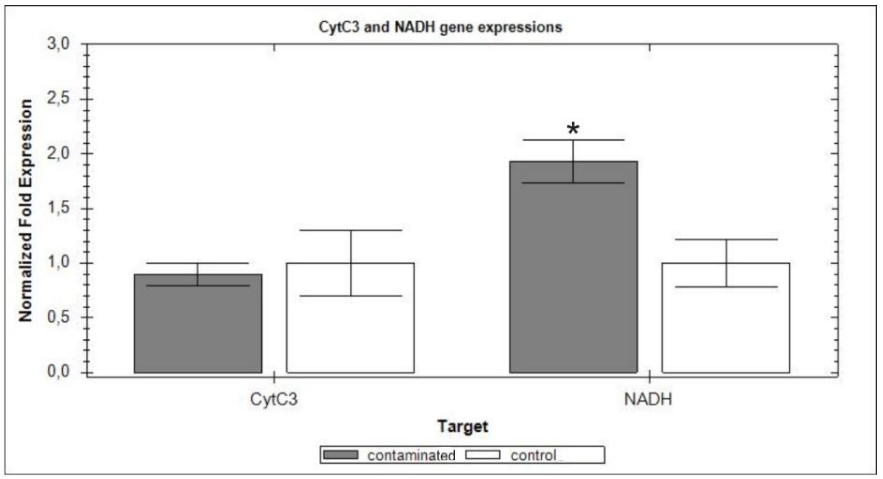
stress (Table 11). The mitochondrial respiratory chain was also highly affected, since several genes involved in this process, had their expression profile altered, probably due to the induction of oxidative stress by metals and radionuclides, present in the contaminated soil. These alterations in the expression levels of many of the genes were described in the literature as being related to hypoxia events, which earthworms may have experienced during this experiment.

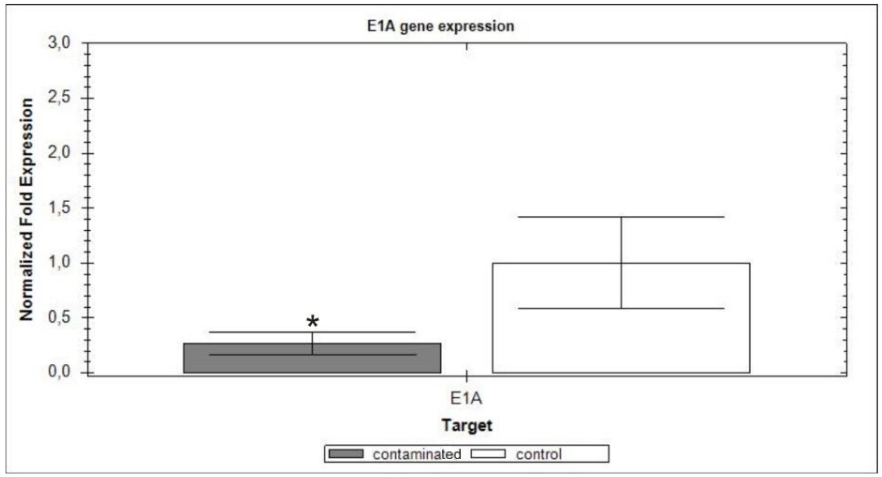
The differencial screening result was confirmed by qPCR as described in section 5.3.5. Up-regulation of chitinase (2.11 normalized fold expression; *p*=0.243), *SET translocation (myeloid leukemia-associated) B* (1.44 normalized fold expression, *p*=0.207), *NADH dehydrogenase subunit 1* (1.93 normalized fold expression; *p*=0.007), were confirmed by RT-qPCR, however statistical differences were only found for the *NADH dehydrogenase subunit 1* gene (Fig. 19). The down-regulation of *cytochrome c oxidase subunit III* (*CytC3*) (0.89 normalized fold expression; *p*=0.764) and *elongation factor 1 alpha* (*E1A*) (0.27 normalized fold expression; *p*=0.025) were also confirmed by RT-qPCR, though statistical differences were only found for the *elongation factor 1 alpha* (*E1A*) gene (Fig. 19).

	Up-regulated genes 14 d	**Down-regulated genes 14 d**	**Up-regulated genes 56 days**	**Down-regulated genes 56 days**
Functional categories				
No hits and unknown (%)	42.85	37.03	55.26	54.05
Cell motility (%)	0	0	0	2.7
Muscle proteins (%)	0	0	0	2.7
Cell signaling (%)	0	7.4	0	2.7
Cytoskeletal proteins (%)	3.57	3.7	2.6	2.7
Homeostasis (%)	3.57	3.7	5.2	8.1
Calcium homeostasis (%)	3.57	0	5.26	0
Immunology (%)	0	0	0	2.7
Cell differentiation (%)	0	3.7	0	0
Redox homeostasis (%)	3.57	0	0	2.7
Metabolism (%)	35.71	14.81	7.84	16.21
Translation (%)	3.57	3.7	2.6	0
Nucleic acid binding (%)	0	14.81	2.6	0
Response to stress (%)	0	3.7	5.26	0
Oxireductase activity (%)	3.57	7.4	7.89	5.4
Nucleosome assembly (%)	0	0	2.6	0
Transporter activity (%)	0	0	2.6	0
Clones sequenced	28	27	38	37

Table 11: General characteristics of the 4 SSH libraries from *Eisenia andrei* exposed to the contaminated soil from the Cunha Baixa uranium mine.







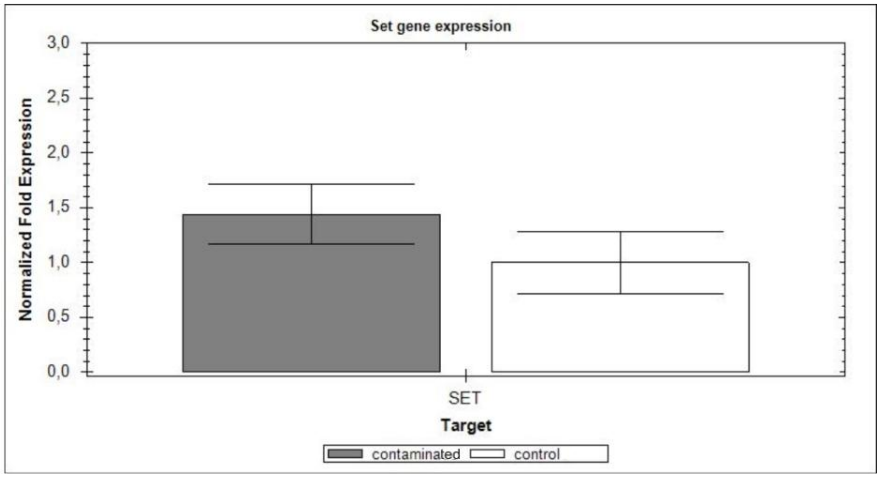


Figure 19: *Chitinase, elongation factor 1 alpha (E1A), NADH dehydrogenase subunit 1, SET translocation (myeloid leukemia-associated) B, cytochrome c oxidase subunit III* (*CytC*3) gene expression analysis in *Eisenia andrei* exposed to LUFA 2.2 in the reference area (white) and exposed to soil B in the contaminated area (dark grey). Data are shown as average ± standard error. (*) stands for statistical significant differences ($p < 0.05$).

5.4.1 Metabolism

An EST coding for a protein with homology for *glyceraldehyde-3-phosphate dehydrogenase (GADPH)* (Table 12) was found to be up-regulated after 14 days of exposure to soil B (Table 12). GAPDH is a glycolytic enzyme responsible for energy production in the cytosol. Yet, recent studies have shown that GAPDH participates in other physiological processes, such as nuclear tRNA transport, apoptosis, DNA repair, DNA replication, tumorigenesis, tumour progression and cell survival (Colell et al. 2009; Higashimura et al. 2011). Despite its use as a housekeeping gene in many studies, GAPDH expression is up-regulated by insulin, calcium, and hypoxia (Higashimura et al. 2011). Another up-regulated EST, with homology with *FIG* superfamily, FIG, FBPase/IMPase/glpX-like domain, was also identified (Table 12) in the same earthworms. The FIG is a superfamily of metal-dependent phosphatases that bind various substrates and participate in processes such as eukaryotic signaling and gluconeogenesis (York et al. 1995). Gluconeogenesis is a process that occurs during starvation (Berg et al. 1995). These results are in agreement to what was observed by Lourenço et al. (2012), in a study where earthworms exposed *in situ* to the same soil that was used in the present study, showed loss of biomass, meaning that earthworms were probably starving. Another EST with homology with the *Nucleoside diphosphate kinases super family*, was also found to be up-regulated (Table 12), after 14 days of exposure to soil B. Rajoutte and Couture (2002) observed that growth rate in fish exposed to metals was lower, and that the high tissue activities of nucleoside diphosphate kinase, an indicator of biosynthesis, indicated an increased rate of protein turnover and suggested a bioenergetic cost of metal exposure. These observations may also be true for the experimental conditions described here; however, it would be necessary to confirm the tissue levels of this protein in the exposed earthworms. Two other EST's identified in this library have homology with *DOMON-like domain of copper-dependent monooxygenases* (Table 12). This domain, also referred as DOH, may be associated with cytochromes or enzymatic domains whose activity is involved in redox or electron transfer reactions (Iyer et al. 2007). Metals participate in these reactions in which they change oxidation states, possibly leading to increased toxicity (Carter 1995). Regarding the down-regulated genes library, an EST was found with homology for *ATP synthase F_0 subunit 6*, after 14 days of exposure (Table 12). The mitochondrial ATP synthase is composed by a catalytic F_1 fraction connected by two stalks with a membrane-embedded F_0 fraction (Houštěk et al. 2006). Houštěk and co-authors (2006) showed that cells with quantitative defects of ATP synthase, evidenced elevated levels of ROS and up-regulation of antioxidative defense components.

Table 12: Classification of differentially expressed ESTs identified from the earthworms *E. andrei*, after 14 days of exposure to the reference soil LUFA 2.2 and to the contaminated soil from the abandoned uranium mining area.

Clone	Species	Accession no.	Putative identity	Blast E-value	RPS-BLAST E-value
Cytoskeletal					
I3	*Lumbricus terrestris*	P92176.1	Actin-2 (similar to Beta actin)	7.00E-12	-
Homeostasis					
A12	*Eisenia andrei*	ACL14179.2	ferritin	3.00E-13	-
Calcium					
F1	*Lumbricus terrestris*	CAD29318.1	Sarcoplasmic calcium binding protein (SCBP) 2	3.00E-21	-
Redox homeostasis					
H14	*Tribolium castaneum*	XP_972053.2	similar to protein disulfide isomerase A6	9.00E-50	-
Metabolism					
J5	*Haemaphysalis*	BAC06447.1	chitinase	1.00E-12	-
C14	-	cl00289	FIG super family, FIG, FBPase/IMPase/glpX-like domain	-	1.79E-04
I4	-	cl09501	phosphatidylserine decarboxylase	-	3.35E-06
C17	*Pleurodeles waltl*	AAL69646.2	glyceraldehyde-3-phosphate dehydrogenase (GADPH)	2.00E-23	-
D5	-	cd09631	DOMON-like domain of copper-dependent	-	7.57E-10
A4	*Saccoglossus kowalevskii*	XP_002733045.1	proteasome beta 7 subunit-like	4.00E-15	-
C9		cl00335	NDPk super family, Nucleoside diphosphate kinases	-	1.63E-03
C1	*Ciona intestinalis*	NP_001108099.1	chitinase	3.00E-13	-
D11	-	cd09631	DOMON-like domain of copper-dependent	-	2.24E-08
J1	-	cl04394	BRICHOS superfamily domain protein	-	5.74E-06
Oxireductase					
A15	-	cl00388	Thioredoxin_like super family	-	1.18E-03

Translation					
H11	*Arenicola marina*	ABW23182.1	ribosomal protein rpl27a	3.00E-33	-
Down-regulated genes					
Cell signaling					
C16	*Lumbricus terrestris*	AAB94561.1	Chemoattractive glycoprotein ES20	2.00E-15	-
C4	-	cl06622	MNNL super family, N terminus of Notch ligand	-	2.48E-03
Cytoskeletal proteins					
C19	*Anolis carolinensis*	XP_003215273.1	actin-related protein 2-like isoform 2	1.00E-59	-
Homeostasis					
B6	*Eisenia fetida*	ABW04906.1	lumbrokinase	4.00E-30	-
Cell differentiation					
E16	*Ornithorhynchus*	XP_001511655.1	similar to leukocyte cell derived chemotaxin 1	8.00E-03	-
Metabolism					
A1	*Apis mellifera*	XP_392257.1	sodium/potassium-transporting ATPase subunit beta-2-like	5.00E-14	-
E14	-	cl04394	BRICHOS superfamily domain protein	-	2.85E-04
C6	*Lumbricus terrestris*	NP 008244.1	ATP synthase F0 subunit 6	4.00E-47	-
B2	-	cl04394	BRICHOS superfamily domain protein	-	2.83E-07
Translation					
F6	*Sipunculus nudus*	ABW90381.1	putative ribosomal protein S18	2.00E-28	-
Nucleic acid binding					
A202	*Saccoglossus*	XP_002733487.1	topoisomerase (DNA) III beta-like	4.00E-33	-
E4	*Hirudo medicinalis*	ABC60437.1	putative elongation factor 1 alpha	1.00E-99	-
A10	*Eisenia sp.*	ABI13234.1	elongation factor-1 alpha	7.00E-20	-
I32	*Hirudo medicinalis*	ABC60437.1	putative elongation factor 1 alpha	1.00E-99	-

Response to stress					
I92	*Lumbricus terrestris*	1X9F_A	hemoglobin dodecamer from Lumbricus erythrocruorin	1.00E-25	-
Oxireductase					
B13	*Lumbricus terrestris*	CAI84975.1	NADH dehydrogenase subunit 2	2.00E-11	-
G4	-	cl02795	Cyt_c_Oxidase_VIc super family, Cytochrome c oxidase subunit VIc.	-	8.01E-18

This was also observed in this work, as some genes with antioxidative defense functions were up-regulated after this exposure period. Also after 14 days of exposure to the contaminated soil, an EST with homology with the *sodium/potassium-transporting ATPase subunit beta-2*, was found to be down-regulated (Table 12). This sodium pump is expressed in all animal tissues, and used in many cellular processes, such as osmoregulation, generation of plasma membrane potential and maintenance of intracellular pH and Ca^{2+} concentration, and excitability in muscle fibers (Tokhtaeva et al., 2009). Therefore the down-regulation of these pumps may lead to severe cell damage and ultimately to cell death.

After 56 days of earthworms exposure to soil B, all the EST's found to be up-regulated belonged to the *BRICHOS superfamily* (Table 13). The BRICHOS domain has been found in proteins with a wide range of functions and disease associations in humans (Hedlund et al., 2009). Regarding down-regulated genes, an EST was found with homology for *phosphoenolpyruvate carboxykinase* (Table 13), which is an enzyme involved in the process of gluconeogenesis (Yang et al., 2009). Kim et al. (2011) found that mRNA levels of *phosphoenolpyruvate carboxykinase (PEPCK)* decreased dramatically upon UV and ionizing radiation exposure. Moreover, they also observed that *PEPCK* promoter activities were also suppressed by exposure to ionizing radiation, suggesting that *PEPCK* gene transcription was down-regulated as a consequence of DNA damage. This might be the case in the present study, since in another study performed under similar conditions (Lourenço et al., 2012), where earthworms were exposed to the same contaminated soil, containing radionuclides (a source of ionizing radiation), DNA damages were recorded after 56 days of exposure. An EST encoding a protein with homology with the *mitochondrial ATP synthase b chain* (Table 13) was also found to be down-regulated. The mitochondrial ATP synthase b chain is a subunit located in the proton – translocating, membrane – traversing F_0 sector of the mitochondrial F_0F_1 ATP synthase (Burger et al., 2003). As referred earlier, the down-regulation of this gene, can interfere with the assembly of this complex, which in turn will cause quantitative defects of ATP synthase in cells, probably contributing for the elevation of ROS levels (Houštěk et al., 2006), and also other serious effects, including depletion of ATP production which will compromise all cellular processes and ultimately cause cell death.

Table 13: Classification of differentially expressed ESTs identified from the earthworms E. *andrei*, after 56 days of exposure to the reference soil LUFA 2.2 and to the contaminated soil from the abandoned uranium mining area.

Clone	Species	Accession no.	Putative identity	Blast E-value	RPS-BLAST E-value
Cytoskeletal proteins					
3F13	*Lumbricus terrestris*	P92182.1	Actin-1	8.00E-102	-
Homeostasis					
3G3	*Eisenia fetida*	AAN75575.1	ARSP1 precursor	5.00E-13	-
3G7	*Eisenia fetida*	ABW04905.1	lumbrokinase	8.00E-12	-
Calcium homeostasis					
3A2	-	cd00051	EF-hand, calcium binding motif	-	8.98E-06
3A15	-	cd00051	EF-hand, calcium binding motif	-	8.98E-06
Metabolism					
3B14	-	cl04394	BRICHOS superfamily domain protein	-	1.08E-05
3A12	-	cl04394	BRICHOS superfamily domain protein	-	2.62E-03
3G12	-	cl04394	BRICHOS superfamily domain protein	-	2.10E-03

Oxireductase activity					
3E14	*Aporrectodea longa*	ACR57130.1	NADH dehydrogenase subunit 1	3.00E-64	-
3H12	*Lumbricus terrestris*	CAI84974.1	NADH dehydrogenase subunit 4	3.00E-63	-
3H10	*Aporrectodea longa*	ACR57130.1	NADH dehydrogenase subunit 1	3.00E-83	-
Translation					
3B4	*Phoronis muelleri*	ACD65142.1	putative 40S ribosomal protein RPS9	8.00E-57	-
Nucleic acid binding					
3E6	*Hirudo medicinalis*	ABC60437.1	putative elongation factor 1 alpha	2.00E-116	-
Response to chemical stimulus					
3G15	*Lumbricus terrestris*	P02218.2	Chain B, Hemoglobin Dodecamer Lumbricus	6.00E-39	-
3I4	*Lumbricus terrestris*	AAC14535.1	hemoglobin chain d1	2.00E-26	-
Nucleosome assembly					
3C13	*Salmo salar*	ACH70631.1	SET translocation (myeloid leukemia-associated) B	2.00E-69	-
Transporter activity					
3H11	*Monodelphis*	XP_001381724.1	similar to heart-type fatty acid-binding protein	5.00E-06	-
Down-regulated genes					
Cell motility					
4J3	*Harpegnathos saltator*	EFN81183.1	Tensin-1	2.00E-19	-
Muscle proteins					
4E15	*Saccoglossus*	XP_002741727.1	ankyrin repeat domain 2-like, partial	9.00E-43	-

Cell signaling					
4C13	*Acromyrmex echinatior*	EGI57728.1	Cell surface glycoprotein 1	1.00E-15	-
Cytoskeletal proteins					
4I2	*Sepia officinalis*	AEE87267.1	beta-actin	1.00E-15	-
Homeostasis					
4J1	*Lymnaea stagnalis*	P42577.2	Soma ferritin	1.00E-24	-
4A14	*Eisenia andrei*	ACL14179.2	ferritin	2.00E-15	-
4J15	*Eisenia fetida*	AAR13225.1	lumbrokinase-4 precursor	3.00E-69	
Metabolism					
4F8	*Caenorhabditis*	XP_003118071.1	CRE-CHT-1 protein	2.00E-13	-
4A1	*Aedes aegypti*	XP_001651134.1	mitochondrial ATP synthase b chain	2.00E-26	-
4G10	*Crassostrea gigas*	CAJ28913.1	phosphoenolpyruvate carboxykinase	3.00E-85	-
4I11	*Anolis carolinensis*	XP_003220815.1	l-lactate dehydrogenase B chain-like	3.00E-64	-
4E13	-	cl04394	BRICHOS super family	-	3.96E-04
4H15	-	cl10447	GH18 (glycosyl hydrolase, family 18) type II chitinases	-	3.67E-18
Immunology					
4B3	*Eisenia fetida*	Q3LX99.1	lysenin-related protein 3	1.00E-108	-
Redox homeostasis					
4A5	*Brugia malayi*	XP_001898144.1	Probable protein disulfide isomerase A6 precursor	7.00E-06	-
Oxireductase activity					
4D10	*Lumbricus terrestris*	NP_008241.1	cytochrome c oxidase subunit III	8.00E-73	-
4B11	*Ixodes scapularis*	XP_002435711.1	cytochrome C oxidase, subunit VIa/COX13, putative	3.00E-30	-

5.4.2 Calcium homeostasis

At both exposure periods (14 and 56 days) the genes involved in calcium homeostasis were found to be up-regulated. After 14 days of exposure, an EST coding for a protein similar to a *sarcoplasmic calcium binding protein (SCBP) 2* (Table 12) was identified. This protein has the important function of buffering calcium to protect cells against massive Ca^{2+} influx (Hermann and Cox, 1995). Ca^{+} overloads may be lethal to the cell since it would rapidly activate a variety of degradative enzymes in an uncontrolled fashion, which could lead to apoptosis or necrosis (Rasola and Bernardi, 2011). After 56 days of exposure, two EST's with *EF-hand calcium binding motifs* (Table 13), could be identified. The EF-hand motif is the most common calcium-binding motif found in proteins (Lewit-Bentley and Réty, 2000). Two primary classes of EF-hand proteins are known: Ca^{2+} sensors, which transduce Ca^{2+} signals, and Ca^{2+} signal modulators, which modulate the shape and/or duration of Ca^{2+} signals or participate in Ca^{2+} homeostasis (Nelson et al., 2002). The activation of Ca^{+} metabolism and homoeostasis mechanisms can protect cells against massive calcium influx. The influx of calcium may be due to the presence of metals in the cells (namely, Zn and Pb)(Morgan and Morgan, 1990; Kiewiet and Ma, 1991), since calcium is known to be involved in the sequestration and elimination of various metals through the chloragogenous tissue (Spurgeon and Hopkin, 1996). Indeed the study performed by Lourenço et al. (2012), showed that earthworms, exposed to the contaminated soil, have bioaccumulated metals, displaying significant higher concentrations of all the metals analyzed, when compared to earthworms exposed to LUFA 2.2 soil. In the chloragogeneous tissue, metals may be detoxified by binding to phosphate ligands in chloragocyte granules in direct exchange with calcium (Spurgeon and Hopkin, 1996). In another study where a histopathological analysis was conducted in organisms that were exposed to metal contaminated soils (Lourenço et al., 2011a), the degradation of the chloragogeneous tissue was clearly observed. Together, these observations strongly support the results herein reported that show, that the overexpression of these genes may be an attempt by the cells to protect them against massive Ca^{+} influx triggered by the bioaccumulation of metals.

5.4.3 Homeostasis

After 14 days of exposure to soil B, an EST with homology for *ferritin* (Table 12), was identified in the up-regulated genes library. Ferritin is an important protein responsible for maintaining of the delicate intracellular iron balance (Orino et al., 2001). It can serve as a cytoprotective protein,

minimizing oxygen free radical formation by sequestering intracellular iron, which in turn protects the cell against oxygen free radical-mediated damage (Orino et al., 2001). Balancing the deleterious and beneficial effects of iron is an essential aspect for cell survival (Orino et al., 2001).

After 56 days of exposure, EST's with homology for *soma ferritin* and *ferritin* were found to be down-regulated (Table 13). Iron plays a central role in protecting the organisms from hypoxemia as it is incorporated in the newly synthesized hemoglobin throughout erythropoiesis (Robach et al., 2007). Robach et al. (2007) showed that, in humans, this essential adaptive response to hypoxia is associated with loss of iron, indicated by the down-regulation of iron proteins, like for example ferritin. A similar process may have occurred in the organisms used in this study.

5.4.4 Oxireductase activity and redox homeostasis

An EST encoding a protein with homology for the *Thioredoxin super family*, as well as an EST involved in redox homeostasis, with homology for *disulfide isomerase A6 (PDIA)*, were up-regulated after 14 days of exposure (Table 12). Disulfide isomerase A6 (PDIA), is a member of the thioredoxin superfamily with chaperone and anti-chaperone activity (Xiao, 2005). Members of the thioredoxin superfamily take an active part in scavenging reactive oxygen species (ROS), thus playing an essential role in maintaining the intracellular redox status (Ahsan et al., 2009). Regarding the down-regulated genes library, an EST with homology for *NADH dehydrogenase subunit 2* was identified (Table 12). The NADH dehydrogenase subunit 2 (ND2) is part of the NADH dehydrogenase complex (or mitochondrial respiratory complex I) that catalyzes the oxidation of NADH by ubiquinone (Nosek and Fukuhara, 1994; Piruat and López-Barneo, 2005). Piruat and Lopéz-Barneo (2005) observed that hypoxia may induce the down-regulation of the mRNAs encoding complex I proteins. Their data suggest that regulation of the mtDNA-encoded complex I subunits participates in mitochondrial O_2 sensing, and that could be an adaptive mechanism during hypoxia. The down-regulation of ND2 may cause a complex I dysfunction, which in turn may increase ROS levels (Sharma et al., 2009), causing oxidative damages. An EST with homology for *Cyt_c_Oxidase_VIc super family, Cytochrome c oxidase subunit Vic*, was also found to be down-regulated after 14 days of exposure (Table 12). Cytochrome c oxidase (CytOX) is the terminal oxidase of the mitochondrial electron transport chain, which catalyzes the reduction of the oxygen (O_2) to water (Vijayasarathy et al., 2003). The VIc subunit belongs to the complex IV of the electron transport chain and is encoded by the nuclear genome, synthesized in the cytosol and imported into mitochondria (Vijayasarathy et al., 2003). Vijayasarathy and co-authors (2003)

observed that hypoxia induces a coordinated down-regulation of both nuclear and mitochondrial genes, showing that hypoxia is, at least, one of the modulation factors affecting CytOX gene expression. Down-regulation of complex IV expression has been shown to enhance oxidant production and cell death (Zhang et al., 2002).

After 56 days of exposure to soil B, EST's with homology for *NADH dehydrogenase subunits 1 and 4 (ND1 and ND4)* were found to be up-regulated (Table 13). Ghosh et al. (2008) observed that cells exposed to chronic oxidative stress, evidenced an increase in expression of these subunits. Their results indicate that alteration in mitochondrial gene expression is mediated through oxidative stress. NADH dehydrogenase subunit 1 was found 2 times in this library, showing its strong up-regulation. Another EST with homology with disulfide isomerase A6 precursor was down-regulated (Table 13). As referred earlier, protein disulfide isomerase A6, plays an essential role in maintaining the intracellular redox status (Ahsan et al., 2009), thus the down-regulation of its precursor may lead to a decreased production of this protein, lowering the defense mechanisms against oxidative stress. Two EST´s with homology for *cytochrome c oxidase subunit III and cytochrome c oxidase, subunit VIa/COX 13* were also found to be down-regulated (Table 13). The subunits III and VIa/COX13 of the cytochrome c oxidase also belong to the complex IV of the electron transport chain, referred earlier for the VIc subunit. However, the subunit III is encoded by the mitochondrial genome while subunit VIa is encoded by the nuclear genome (Fontanesi et al., 2006). The down-regulation of these genes may cause alterations in the assembly process of cytochrome c oxidase complex, producing inefficient enzymes that could lead to an increase in the production of ROS (Fontanesi et al., 2006).

5.4.5 Response to chemical stimulus and stress

After 14 days of exposure to soil B, a gene with homology for a *hemoglobin dodecamer from Lumbricus erythrocruorin*, was found to be down-regulated (Table 12). Erythrocruorins are highly cooperative giant extracellular respiratory complexes found in annelids, where they have the same function as the red blood cells (Strand et al., 2004). Unlike most hemoglobins, erythrocruorins are not packaged into cells, but are freely dissolved in the blood of annelids (Strand et al., 2004). In addition to O_2 transport and storage, they are involved in O_2 scavenging, O_2 sensing, oxidase and peroxidase activities, the latter related to detoxification (Weber and Vinogradov, 2001). The erythrocruorins were also described by Harrington (2007) as exhibiting unusually highly positive redox potential, supporting the idea that globins could share a protective

role against oxidative stress (Nishi et al., 2011). The down-regulation of this gene may impair the formation of the erythrocruorins complex possibly causing a depletion of the amount oxygen distributed to cells, which can lead to hypoxia. Moreover, it may decrease the ability of these organisms to fight oxidative stress, caused by the exposure to metals and radionuclides.

After 56 days of exposure, EST's with homology for *Chain B, hemoglobin dodecamer from Lumbricus erythrocruorin* and *hemoglobin chain d1* were found to be up-regulated (Table 13). The up-regulation of these genes was probably an attempt to increase the amount of oxygen delivered to cells, in order to reverse an eventual hypoxia event. This may be an attempt of earthworms to fight oxidative stress and adapt to the surrounding environment by reestablishing homeostasis. Moreover, Robach et al. (2007) states that, in humans, in the erythropoietic compartment, where 70% of the iron is stored, hypoxia stimulates erythropoiesis and promotes hemoglobin synthesis. A similar process may have occurred in earthworms, which may help to explain the expression profile of these genes.

5.4.6 Nucleic acid binding

A gene with homology with the *topoisomerase (DNA) III beta (Top3)* (Table 12) was down-regulated after 14 days of exposure to soil B. This gene encodes one of the two mammalian type IA topoisomerases (Kwan et al., 2003), which have been implicated in the repair of dsDNA breaks and in the resolution of stalled or damaged DNA replication forks (Mohanty et al., 2008). This protein is also involved in the activation of cellular responses to DNA damage, thus playing an important role in the maintenance of genomic stability (Mohanty et al., 2008). Mohanty et al. (2008) observed that cell deficient in TOP3, displayed increased genotoxic sensitivity, namely to UV-B, hydroxyurea and ionizing radiation. This is in agreement to what was observed in the study performed by Lourenço et al. (2012), where DNA damages were detected by the comet assay in earthworms also exposed *in situ* to the same soil used in this study. Likewise, genes with homology for the *elongation factor 1 alpha* from different organisms, were also found to be down regulated (Table 12). This gene was found 3 times in this library, showing that it is strongly down-regulated. The eukaryotic elongation factor 1 alpha (eEF1A) is required for the elongation step of translation, however, has also other functions unrelated to protein synthesis (Byun et al., 2009). Byun et al. (2009) identified and validated eEF1A as a reliable biomarker of cell senescence, induced by ionizing radiation (IR). This gene was down-regulated in senescent cells induced not only by IR but also by hydrogen peroxide exposure and low doses of chemotherapeutic drugs

(Byun et al., 2009). As described in the Materials and Methods section, the contaminated soil (soil B), used in this experiment, has high levels of radionuclides, which probably caused the down-regulation of this gene. Moreover, Lourenço et al. (2012), showed that earthworms, have bioaccumulated radionuclides showing higher levels of all the isotopes analyzed, when compared to earthworms exposed to the control soil.

After 56 days of exposure to soil B, an EST with homology for *elongation factor 1 alpha* was found in the up-regulated genes library (Table 13). Chen et al. (2000) showed that the increased level of expression of *elongation factor 1 alpha* is an immediate early event of apoptosis caused by oxidative stress and that elongation factor 1 alpha protein is an immediate early biochemical marker of oxidative-stress-associated cell suicide (Chen et al., 2000). Lourenço et al. (2012) reported a decrease of DNA damage after 56 days of exposure suggesting the elimination of cells severely damaged by the exposure to metals and radionuclides, by apoptosis.

5.4.7 Nucleossome assembly

A gene with homology with *SET translocation (myeloid leukemia-associated) B*, was found up-regulated, after 56 days of exposure (Table 13). SET (also known as template-activating factor I beta (TAF-Ib) interacts with several proteins complexes, indicating its diverse functions that include Granzyme A-induced apoptosis, chromosome remodeling, transcriptional regulation, mRNA stabilization and cell cycle regulation (Kandilci and Grosveld, 2005). Canela et al. (2003) observed that the up-regulation of SET induced an increase in the number of cells at G_2/M and a decrease in the number of cells in the G_1 phase. Similar results were reported by Lourenço et al. (2012), that registered an increase of coelomocytes in the G2/M phase of the cell cycle after 56 days of exposure. This cell cycle arrest may be a consequence of DNA damages caused by the exposure to metals and radionuclides. The presence of DNA damages in earthworms coelomocytes, after 56 days of exposure to the same contaminated soil used in the present study, was also confirmed by Lourenço et al. (2012). Moreover, this gene is an oncogene which can form chimeric proteins when fused with other genes, as observed for instance in acute undifferentiated leukemia (Adachi et al., 1994; Canela et al., 2003), and it also seems to be involved in other cancer types (Jiang et al., 2011). This may be an important aspect, since this study was performed in a soil from an uranium mining area, contaminated with uranium and its daughter radionuclides, where earthworms are exposed not only, but also, to ionizing radiation, which has the potential to promote cell transformation. The up-regulation of this gene was confirmed by RT-qPCR using *28S*

rRNA as a reference gene, however no significant differences of gene expression were found between exposed and control organisms. Despite that fact, attention should be given to this gene since, to the best of our knowledge, this is the first time that the presence of *SET* gene is reported in this type of studies.

5.4.8 Immunology

A gene with homology for a *lysenin-related protein 3* was found to be down-regulated after 56 days of exposure (Table 13). Lysenin and lysenin related proteins, present in the coelomic fluid of earthworms, are part of the defense mechanisms of these organisms, since they are pore-forming toxins that specifically bind sphingomyelin, which is present in the plasma membrane of various cells (Ishitsuka and Kobayashi, 2004), except in invertebrates (Kobayashi et al., 2000). These characteristics confer to these proteins hemolytic and cytotoxic properties (Ishitsuka and Kobayashi, 2004). Lysenin is secreted from dorsal pores in response to mechanical or chemical stimuli, and thus, might contribute to defense functions against some vertebrate animals (Kobayashi et al., 2000). Consequently the down-regulation of this gene may decrease earthworm's defenses. An analysis of *Eisenia fetida* proteome during cadmium exposure performed by Wang et al. (2010b), also reported a down-regulation of lysenin protein in these organisms. The exposure to metals and radionuclides has been shown to negatively influence the immune system of invertebrates (Ishitsuka and Kobayashi, 2004; Lourenço et al., 2011b; Lourenço et al., 2012), which potentially affected the expression of this gene.

5.5 Conclusions

The use of SSH technique allowed us to obtain a global perspective of the gene expression profile of earthworms exposed to metals and radionuclides, when compared to those exposed to the control soil, highlighting the complexity of the responses to a mixture of these inorganic stressors, under environmental conditions. Although the fact that transcriptional changes may not reflect accurately the changes at a protein level, preventing mechanistic interpretations, they are nonetheless a response of the organism to the environmental stress imposed. Therefore, these studies are important to provide indications at a molecular level of the effects of the exposure to toxicants, but most importantly, to the development of more sensitive and specific biomarkers that may be employed as early warning signals for the exposure to toxicants.

The results herein reported clearly demonstrated that the exposure to the mine soil contaminated with metals and radionuclides altered the expression of some genes involved in key physiological functions associated with the response to oxidative stress, namely oxireductase activity, redox homeostasis and response to chemical stimulus and stress. Moreover, the alteration of the expression pattern of many of the identified genes, strongly support the interpretation of the results in previous studies performed by our team, with the same organisms (Lourenço et al., 2011a; Lourenço et al., 2012). The significant alteration of the expression of *NADH dehydrogenase subunit 1* and *elongation factor 1 alpha*, may indicate that these genes are in the basis of alterations, such as the DNA damages, observed in a similar study performed by Lourenço et al., (2012). Therefore, studies like this one and others that were referred along the present work, employing these methodologies, are indispensable to show that gene expression profiling is of utmost importance to assess underlying mechanisms of toxicity, which cannot be detected by standard ecotoxicological tests.

Moreover, this approach allowed the identification of an EST with homology with the oncogene *SET*, described for the first time in this type of studies and, to the best of our knowledge, for the first time in earthworms. The functions attributed to this gene and associated to the fact that it is up-regulated, renders its detection very interesting and worthy of attention in future studies, to clarify its involvement in the toxicity of metals and radionuclides.

5.6 References

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CHAPTER 6 - METALS BIOACCUMULATION, GENOTOXICITY AND GENE EXPRESSION IN THE EUROPEAN WOOD MOUSE (*APODEMUS SYLVATICUS*) INHABITING AN ABANDONED URANIUM MINING AREA

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6.1 Abstract

Genotoxic effects caused by the exposure to wastes containing metals and radionuclides were investigated in the European wood mice (*Apodemus sylvaticus*). The animals were captured nearby an abandoned uranium mining site. DNA damages were assessed by comet assay; gene expression and single nucleotide polymorphisms (SNPs) were assessed by Real-Time PCR and melt curve analysis, respectively. The bioaccumulation of metals in the liver, kidney and bones was also determined to help clarify cause-effect relationships. Results confirmed the bioaccumulation of cadmium and uranium by organisms exposed to uranium mining wastes. *P53* gene was found to be significantly up-regulated in the liver of those organisms and SNPs in the *Rb* gene were also detected in the kidney. Our results showed that, the exposure to uranium mining wastes caused serious DNA damages leading to genomic instability, evidenced by the significant increase in DNA strand breaks and disturbance of *p53* gene expression. These effects can have serious consequences, since they may contribute for the emergence of serious genetic diseases. Since mice are often used as bioindicator species for the evaluation of risks of environmental exposure to wildlife and to humans, the results observed raised concerns about the potential risks to human populations living nearby uranium mining areas. Nevertheless, since there are remarkable differences in exposure pathways of both species, an evaluation using human biological samples and biomarkers should be performed.

Keywords: DNA damages; mutations; gene expression; metals; radionuclides, bioaccumulation

6.2 Introduction

The impact of uranium mining and milling on the environment has long been recognized (Lozano et al. 2000). The extraction of uranium has an important radioactive and chemical impact, since it causes the surface exposure of geologic material rich in uranium and its radioactive descendants, as well as other metals and metalloids (Lozano et al. 2000; Carvalho et al. 2007; André et al. 2009; Pereira et al. 2008; Lourenço et al. 2012). Consequently, even after the cessation of mining activities, the impacts on the environment persist for several decades (Pereira et al. 2006; Lozano et al. 2000), due to weathering conditions. Chronic exposure to such contaminants may result in continued bioaccumulation and has been linked to several harmful effects in living organisms, since they may have mutagenic, carcinogenic and teratogenic properties, among others (Amaral et al. 2007; Lourenço et al. 2012). Data collected from vertebrates living in/exposed to contaminated areas, are of utmost importance since they can bring pertinent information to be used in human risk assessment (Pereira et al. 2006). Furthermore, field studies provide crucial ecotoxicological data, since under laboratory conditions it is difficult to mimic the exposure to complex mixtures of contaminants, synergistic and/or antagonistic effects, as well as the complex interactions between abiotic and biotic factors and their role in mixtures toxicity (Sánchez-Chardi et al. 2007; Pereira et al. 2006; Léon et al. 2007). Environmental exposure of terrestrial vertebrates to non-essential metals, in contaminated areas, is known to be associated with a wide range of toxic effects, at different levels of biological organization (Sánchez-Chardi et al. 2007; Sánchez-Chardi et al. 2009), since these organisms bioaccumulate them in different tissues and organs (Sánchez-Chardi et al. 2007; Marcheselli et al. 2010; Pereira et al. 2006; Amaral et al. 2007)

The monitoring of genotoxic effects in the environment requires the selection of representative organisms as sentinels, as well as the development of suitable and sensitive assays (Festa et al. 2003). Among these organisms, small mammals such as rodents are preferentially selected, since they are considered to be sensitive to contaminants and appropriate bioindicator species for monitoring exposures and effects (Festa et al. 2003; Marcheselli et al. 2010; Beernaert et al. 2007). Studies on small mammals have demonstrated their ability to accumulate a wide spectrum of pollutants, widespread in their distribution areas (Ieradi et al. 1998; Sánchez-Chardi et al. 2007). Additionally, small mammals usually fulfill the criteria to be selected as bioindicator species, namely: high abundance, a relatively small feeding and breeding area and a life expectancy high enough to estimate possible long-term effects (Marcheselli et al. 2010). The ubiquitous wood mouse *Apodemus sylvaticus* is one of the species that meets these criteria

(Marcheselli et al. 2010; Sánchez-Chardi et al. 2007). Although occasionally showing carnivorous behavior, this mouse is mainly a primary consumer and, as such, is often used as a bioindicator of pollution (Sánchez-Chardi et al. 2007; Marcheselli et al. 2010; Rogival et al. 2006; Sánchez-Chardi et al. 2009). This species is exposed to contaminants mainly via inhalation of soil particles and ingestion of contaminated food and water (Marcheselli et al. 2010). Significant correlations between metal contamination (Festa et al. 2003), radioactivity and genetic damage in free-living rodents have been detected (Ieradi et al. 1998), corroborating their sensitivity to these stress agents.

Comet assay is one among other methodologies that is used to detect genetic damage. It is a simple, rapid and sensitive tool to assess DNA damage and repair in individual cells and it has increasingly found application in diverse fields ranging from genetic toxicology to human epidemiology (Dhawan et al. 2009). The determination of DNA damage in sentinel species, can provide information about the genotoxic potential of their habitat at an early stage, allowing the implementation of strategies for the prevention or reduction of deleterious health effects in wildlife species as well as in humans (Dhawan et al. 2009). The exposure to potentially toxic chemical compounds also involve changes in gene expression profiles in receptor organisms (Brulle et al. 2010). Thus, the analysis of changes in gene expression is a powerful tool to diagnose the existence of a stress condition and to analyze the response of organisms (Brulle et al. 2008; Bernard et al. 2010). Some molecular-genetic tools like quantitative reverse transcription PCR (RT-qPCR), widely developed in the last decade, allow the analysis of transcriptomes of stressed organisms, exposed in laboratory or under field conditions (Brulle et al. 2010; Pirooznia et al. 2007; Lee et al. 2005). Gene expression analysis by qRT–PCR allows the accurate and sensitive measurement of gene expression levels (Willems et al. 2008). The environmental exposure to genotoxic contaminants, may also cause mutations and/or single nucleotide polymorphisms (Pfeifer 2010). Therefore their evaluation is useful to evaluate the genomic instability and also the predisposition for the development of genetic diseases, as a consequence of exposure to genotoxic contaminants (Pfeifer 2010).

This study aimed to evaluate the genotoxic effects occurring in the European wood mouse, inhabiting the deposition area of uranium mining wastes, rich in metals and radionuclides. Moreover, changes in the expression level and the presence of SNPs in the tumour suppressor genes *P53* and *Rb* in target organs were also investigated, since it is well recognized that metals and radionuclides can have mutagenic and carcinogenic properties (Amaral et al. 2007; UNSCEAR 2000). *Rb* and *P53* genes are crucial in the cellular response to a range of environmental and

intracellular stresses (Meek 2004). They act mainly in the control of the G1/S checkpoint of the cell cycle, eliciting cell cycle arrest to allow for DNA repair and/or apoptosis in response to a variety of genotoxic stresses (Meek 2004; Genovese et al. 2006). Data on the bioaccumulation of metals in target organs was also important to clarify cause-effect relationships.

6.3 Materials and Methods

6.3.1 Study site

The Cunha Baixa uranium mine (coordinates: 40.571393 N 7.753322 W), was one of the largest uranium mines in Portugal (Carvalho et al. 2009). The mine is located southeast of Viseu, near the Cunha Baixa village. The underground and open pit mining works, carried out between 1970–1993, produced about 1000 t of U_3O_8 and one million tons of waste materials which were disposed in a dump surrounding the mine area (Neves et al. 2005). Furthermore, sulfuric acid was used for *in situ* leaching of uranium in underground works as well as in heap leaching of low grade ores at the surface (Pereira et al. 2008; Carvalho et al. 2009; Antunes et al. 2008a). The mine pit water is, therefore, acidic (pH <3.5) and is able to move through permeable heap leaching wastes, following changes in the piezometric level of the underlying aquifer, mobilizing elements and increasing their dispersion (Neves et al. 2005). Since the end of the mining activity, the acidic water from the mine pit has been almost continuously pumped out to treatment plants and treated with burned lime and barium chloride for pH neutralization and radionuclides precipitation, respectively (Pereira et al. 2008; Carvalho et al. 2009). When the maximum capacity of the pond is reached, the treated affluent is discharged in a stream passing nearby, and the sludge formed by the accumulation of the chemical compounds at the bottom is removed and spread near the mine pit. The sludge has high levels of metals (Pereira et al. 2008) and radionuclides from uranium series (Carvalho et al. 2007). Soils from the adjacent areas of the mine pit have suffered several impacts, through the deposition of both tailings and sludge from the wastewater treatment plant (Antunes et al. 2008a; Pereira et al. 2008).

6.3.2 Test organisms and sampling procedure

European wood mice (*Apodemus sylvaticus*) were collected at a pine-oak tree forest, 3 Km apart from the mining area (reference site), and in the surroundings of the mine pit

(contaminated site), which is also a pine-oak tree forest, including tailings and sludge deposition sites (Fig. 20). The reported average home range of *Apodemus sylvaticus* is about 2250 m^2 for non fragmented areas (Mertens et al. 2001; Marcheselli et al. 2010), which is smaller than the surface areas of the chosen study-sites. As so, it was assumed that the wood mice captured fed and live mainly inside the study-sites.

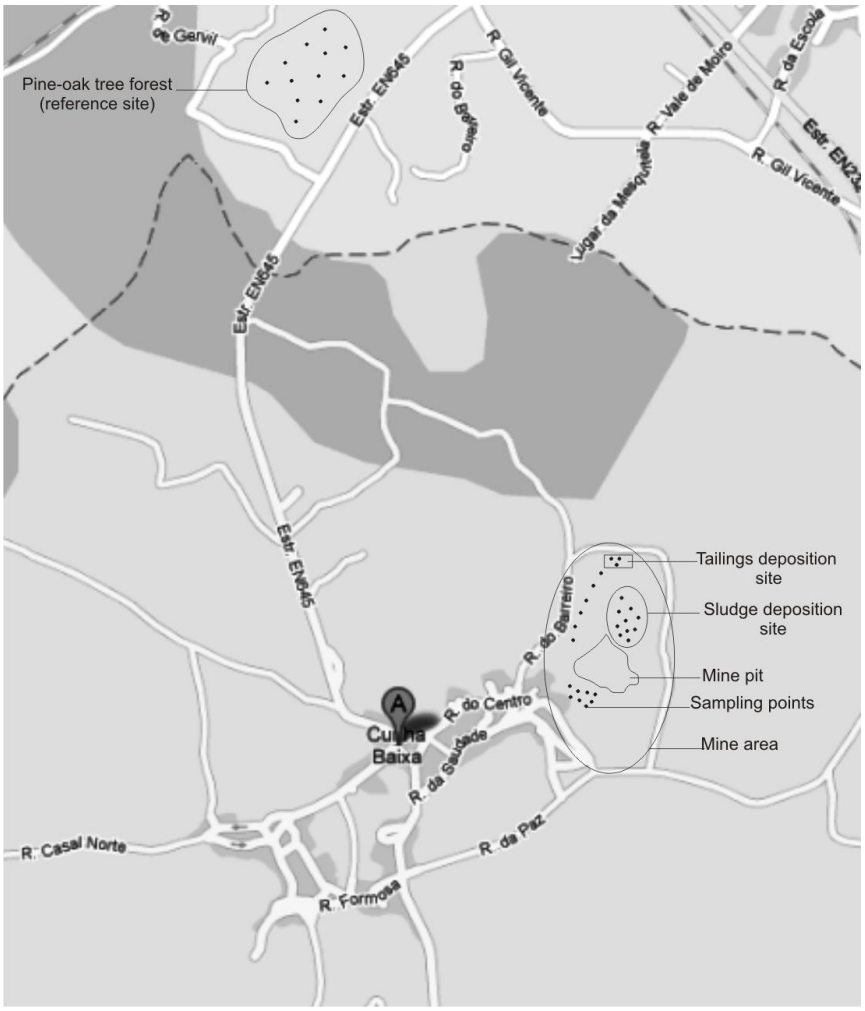

Figure 20: Map of the study area, showing the location of the Cunha Baixa village and the sampling sites. The approximate location of the mine pit and waste deposition areas are also indicated. The sampling points are marked with a black dot

The organisms were captured in 3-day capture periods, using live traps baited with a mixture of canned tuna fish and baby pap. In total 21 individuals were trapped, being 11 from the contaminated site and 10 from the reference site (Table 14).

The captured animals were taken alive to the laboratory, where they were anaesthetized and sacrificed according to the measures established by the Directive 2010/63/EU of the European Parliament and the Council, of 22 of September 2010, for reducing pain, suffering distress or lasting harm to animals (Article 1); Furthermore, one of the authors, which has performed these procedures is certified by national authorities (FI/285/2011) for animal experimentation. After being weighed, the sex of each animal was determined and standard mammal body measurements were performed (Table 14). Liver, kidneys, and bones were dissected and weighted to the nearest 0.1 mg. Blood was extracted using a syringe containing sodium EDTA and preserved at 4ºC with 10% of DMSO until analysis (about 4 hours). A fraction of the liver and kidneys were fixed in RNA later (Qiagen, Germany) and stored at -80ºC, until RNA extraction. The remaining fraction of these organs as well as the bones, were frozen at -20ºC until wet digestion and metal content analysis.

Table 14: Characteristics and standard mammal body measurements of the studied populations

	Reference site	**Contaminated site**
Sample size	11	10
Total weight (g) (Mean ± SD)	26.3 ± 5.6	24.3 ± 5.2
Total length (cm) (Mean ± SD)	9 ± 0.6	8.9 ± 0.8
Length of the rear paw (cm) (Mean ± SD)	2.2 ± 0.2	2.2 ± 0.3
Length of the ears (cm) (Mean ± SD)	1.6 ± 0.2	1.5 ± 0.3
Nº of females	4	3
Nº of males	7	7

6.3.3 Comet assay

For the comet assay, fresh whole blood samples were used directly from the collection tube (11 and 10 samples from the reference and contaminated sites, respectively). The assay was conducted under yellow light, to prevent UV induced DNA damage, and performed with slight modifications of the protocol described by Nogueira et al. (2006): briefly, microscope slides were covered with a first agarose layer, left to dry and a second layer containing the cells was placed on

top. Visual scoring of cellular DNA on each slide was based on the categorization of 100 cells randomly selected. The comet-like formations were visually graded into 5 classes, depending on DNA damage, and scored as described by Garcia et al. (2004). Positive controls were always included, and consisted of cells previously exposed to 200 μM of H_2O_2, for 1 h.

6.3.4 RNA extraction and RT-qPCR

RT-qPCR analyses were performed in kidney and liver samples from 5 organisms, 3 males and 2 females, from each study-site. Total RNA was extracted using PureLink™ RNA Mini Kit (Invitrogen, USA), treated on column with PureLink™ DNase (Invitrogen, USA), quantified using Qubit™ Fluorometer (Invitrogen, USA) and Quant-iT™ RNA Assay Kit (Invitrogen, USA). The extracted RNAs were also checked for quality by visually assessing the ratio of 28S:18S RNA on agarose gel stained with EtBr. First-strand cDNA was synthesized using SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen, USA). First-strand cDNA was synthesized from 2 μg of total RNA in the presence of random hexamer primers. The genes *peptidylprolyl isomerase A*, *beta-actin* and *18S* were used as reference genes for the kidney samples. As for the liver samples, *peptidylprolyl isomerase A* (*Ppia*), *beta-actin* and *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) were used as reference genes. The *cyclin G-associated kinase* (*Gak*) and the *signal recognition particle 72* (*Srp*72) genes were also tested to be used as reference genes, but were not suitable for this experiment. The stability of the reference genes used in this study, was determined using the qbase^{Plus}/qPCR data analysis software in which geNorm software is incorporated. qPCR reactions were performed in a final volume of 20 μl containing 10 μl of SsoFast™ EvaGreen® Supermix (Biorad, USA), 1 μl of diluted (1:10) cDNA and 200 nM primers. A non-template control (NTC) was included in the qPCR analysis to determine the specificity of target cDNA amplification and also a non-RT control (NRT), including RNA instead of cDNA, to determine if the RNAs were contaminated with genomic DNA. The RT-qPCR was performed using the CFX96™ Real-Time System (Biorad, USA) and the following cycling parameters: 98ºC for 30 s and 44 cycles of 5 s at 95ºC and 10 s at 57ºC. Melting curves were performed to identify the presence of primer dimers and to analyze the specificity of the reaction. The amplification efficiency of each primer pair was calculated using a dilution series of cDNA. The primers used in RT-qPCR were designed using *Mus musculus* sequences deposited in the Genbank database and are listed in Table 15. Two sets of primers for the *Rb* gene are listed in Table 15, since one of them (*Rb*1) was not suitable for the gene expression analysis in the liver samples. The primers set *Rb*1 was only used in liver samples

for the detection of mutations in the region between exons 11 and 12 of the *Rb* gene. The relative expression levels of the target genes, were calculated according to the efficiency corrected method described by Pfaffl (2001).

The amplified fragments of the target genes *P53* and *Rb* were also screened for the presence of SNPs. Fragments were amplified as referred earlier in a master mix containing the EvaGreen fluorescent dye (EvaGreen® Supermix (Biorad, USA)), and SNP's were detected by analyzing the EvaGreen melt curve behavior of each amplification product (Temesvári et al. 2011).

Table 15: Sequences of the primers used in RT-qPCR.

Gene	Sequence (5'- 3')
18S	For: ATGGCCGTTCTTAGTTGGTG Rev: CGCTGAGCCAGTCAGTGTAG
Ppia	For: AGCATACAGGTCCTGGCATC Rev: CACCTTCCCAAAGACCACAT
B-actin	For: ACTGGGACGACATGGAGAAG Rev: GGGGTGTTGAAGGTCTCAAA
Gapdh	For: AACTTTGGCATTGTGGAAGG Rev: ACACATTGGGGGTAGGAACA
P53	For: AACCGCCGACCTATCCTTAC Rev: CTTCTGTACGGCGGTCTCTC
Rb1	For: AGAGAGAACGCCACGAAAAA Rev: GATGGCTGATCACTTGCAGA
Rb2	For: TGCATGGCTTTCAGATTCAC Rev: ACAGGGCAAGGGAGGTAGAT

6.3.5 Chemical analysis

The total concentration of Be, Al, Mn, Ni, Cu, Zn, Sr, Cd, Pb and U were determined in the liver, kidneys and bones of the organisms captured in the mine and in the reference area (a total of 21 organisms), by inductively coupled plasma mass spectrometry (ICP-MS) (APHA 1995). The tissues were oven-dried at 105ºC until weight stabilization, and then the weights were recorded to the nearest 0.1mg. Then, they were digested in Teflon vials containing 3 mL suprapur nitric acid (65%, Merck) in a 60ºC sand bath, until there were no solid fragments in the solution; 1.5 mL of suprapur hydrogen peroxide (30%, Merck) was added to the vials and placed again in the sand

bath until the solution became clear. Finally, ultra-pure water was added to a total volume of 5 mL. Reagent blanks were obtained following the same procedure but without the tissues.

6.3.6 Statistical analysis

Regarding chemical analysis and comet assay results, the statistical analysis was performed using one-way ANOVAs and Kruskal-Wallis one-way analysis of variance on ranks, whenever the data failed to meet the normality and homoscedascity assumptions. The data obtained by real-time qPCR, were analyzed using qbase^{Plus} software that runned Mann-Whitney tests on the results obtained. The level of significance defined for all the analyses was 0.05.

6.4 Results and Discussion

The metal content in the analyzed organs revealed that Cd and U were significantly bioaccumulated by mice inhabiting the contaminated site (Table 16). Additionally, these animals also showed significantly higher levels of Cu in the bones (Table 16). Indeed, a study performed by Pereira et al. (2008), showed that the soils from the mine area (designated in that study by A, B, C) where the mice were captured, had higher levels of these metals when compared to the reference site (designated by J in that study). These results are in agreement with the levels found in these organisms. However, the levels of Sr were higher in the organisms from the reference site (Table 16). In the same study published by Pereira et al. (2008), it was determined that the levels of Sr in the reference site were similar to those registered in the mine area. A higher bioavailable fraction of this metal in the reference area may be an explanation for the bioaccumulation differences observed between mice inhabiting the reference and contaminated sites. Another hypothetical explanation may be related to the fact that the intake of uranium is associated with kidney function, increased fractional excretion of calcium and phosphate in urine (Kurttio et al. 2005). Since strontium mimics calcium in the formation of bones, due to their chemical similarity (Dahl et al. 2001), the accumulation of uranium in mice inhabiting the contaminated areas may stimulate the excretion of the strontium accumulated in the bones, explaining the lower levels of this metal found in these animals when compared to those inhabiting the reference area.

As referred, the evaluation of DNA damages in exposed animals was performed using the comet assay. DNA is an important target of environmental stress in terrestrial organisms (Reinecke and Reinecke 2004). The loss of DNA integrity may determine the induction of mutations and other irreversible toxic effects (Kurelec 1993). The results showed that there was a

significant loss of DNA integrity in the organisms living in the mining area (Fig. 21), probably caused by the bioaccumulation of uranium and cadmium and also by the exposure to uranium and its daughter radionuclides. The level of strand breakage in DNA has been proposed as a sensitive indicator of genotoxicity and an effective biomarker in environmental biomonitoring (Shugart and Theodorakis 1998; Shugart 2000). Metals like Cd and U, are known to cause severe damages to organisms, such as, tissue damages (Pereira et al. 2006; Amaral et al. 2007; Lourenço et al. 2011), apoptosis induction (Amaral et al. 2007), DNA damages (Lourenço et al. 2012) and chromosomal aberrations involving gross alterations of the genetic material (Topashka-Ancheva et al. 2003). Moreover, they are capable of inducing redox imbalance in living organisms, leading to the production and accumulation of reactive oxygen species (ROS) inside the cells, causing damages in the DNA molecule, like DNA strand breaks, DNA-protein cross-links, alkali labile sites, and oxidative base modifications (Barillet et al. 2010). In addition, metals can cause direct DNA strand breaks, without any involvement of free radicals, and inhibit DNA repair (Barillet et al. 2010). Uranium extraction also involves the generation of solid residues contaminated with radioelements (U and its daughter radionuclides), which contributes to an extensive contamination of the soil compartment (Pereira et al. 2008; Lourenço et al. 2012). The radionuclides found in this area are mainly alpha emitters (Carvalho et al. 2009). Alpha particles (high energy, relative large mass and momentum, low velocity) have relatively high linear energy transfer (LET) values (Harrison and Day 2008). Clustered DNA damage, along with the degree of complexity of the damage, has been shown to increase with LET (Valentin 2003). The biological consequence depends whether the damage can be repaired and with what fidelity (Harrison and Day 2008). Both laboratory and field studies have shown that sublethal effects at a low level of biological organization like, for example DNA damages, influence energy metabolism, fitness and reproductive success, which leads to population level effects (Jha 2008). As so, the wood mice population living in the contaminated site, may suffer from increased genomic instability, which has been suggested to play an important role in decreased fitness of the population not only in the laboratory studies but also in field conditions (Jha 2008). This will ultimately lead to adverse effects on long-term population survival and, hence deterioration of the ecosystem (Jha 2008). Further studies are required to determine these effects, as they were not the target of this study.

Table 16: Metal content of the organs analyzed (μg.g^{-1} dry weight) (mean ± standard deviation, *n*=11 for the reference site and *n*=10 for the contaminated site).

Sampling sites	Organs	Be	Al	Mn	Ni	Cu	Zn	Sr	Cd	Pb	U
Organs metal content ± SD (μg.g^{-1} dry weight)											
Control site	Bone	UDL	2.95 ± 0.49	1.36 ± 0.24	3.41 ± 0.42	1.66 ± 0.53	162.09 ± 54.9	180.02 ± 46.68	0.1± 0.014	0.27± 0.09	UDL
	Kidney		UDL	5.66 ± 2.19	UDL	14.82 ± 4.35	105.29 ± 36.42	UDL	0.7 ± 0.27	0.33 ± 0.19	0.012 ± 0.0005
	Liver		3.73 ± 2.02	10.97 ± 2.93	0.23 ± 0.14	15.58 ± 1.9	169.54 ± 52.9	0.32± 0.07	0.16 ± 0.06	0.05 ± 0.02	0.004 ± 0.0007
Contaminated site	Bone	UDL	6 ± 4.95	1.35 ± 0.73	3.46 ± 0.58	2.03 ± 0.36(*)	170.02 ± 70.25	85.92 ± 38.14(*)	0.14 ± 0.08(*)	0.29 ± 0.17	0.17 ± 0.16(*)
	Kidney		UDL	6.11 ± 1.38	UDL	16.39 ± 1.95	114.44 ± 32.7	UDL	1.35 ± 0.49(*)	0.25 ± 0.15	0.16 ± 0.15(*)
	Liver		4.22 ± 2.52	9.19 ± 3.83	0.25 ± 0.11	18.94 ± 6.33	163.34 ± 68.57	0.15 ± 0.06 (*)	0.32 ± 0.17(*)	0.04 ± 0.02	0.03 ± 0.04(*)

(*) Statistical significant differences between sampling sites, p <0.05.
UDL: Under Detection Limits

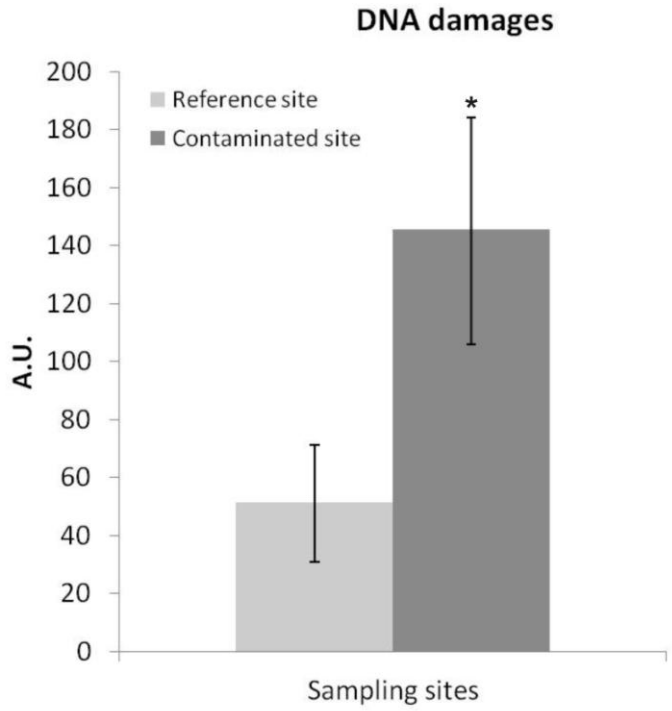

Figure 21: Whole blood DNA integrity in *Apodemus sylvaticus* inhabiting the reference (light grey) and contaminated (dark grey) sites. Data are shown as average ± standard deviation. (*) stands for statistical significant differences ($p < 0.05$)

Gene expression changes are increasingly used to assess the effects of exposure to environmental agents (Banda et al. 2008). Herein the expression of *P53* and *Rb* genes was analyzed in the kidney and liver of mice inhabiting a pine-oak tree forest and compared to that of mice collected at an abandoned uranium mining area. On the kidney samples, no significant differences were found between both sampling sites either for the *P53* (1.58 normalized fold expression; p=0.11) and the *Rb* gene (1.04 normalized fold expression; p=0.84) (Fig. 22). Concerning the liver, no significant differences were found for the *Rb* gene (0.96 normalized fold expression; p=0.34) (Fig. 22); yet, significant differences were found for the *P53* gene (2.32 normalized fold expression; p=0.03) (Fig. 22). The tumor suppressor gene *P53* is a "cellular gatekeeper", playing an important role in the control of cell proliferation, induction of programmed cell death (apoptosis), and cell differentiation (Imazawa et al. 2003). The induction of *P53* can occur in response to a range of genotoxic stresses leading to growth arrest or apoptosis (Meek 2004), by transcriptional regulation of the genes involved in these processes (Gillet et al. 2000). Whereas in normal cells P53 is maintained at an undetectable or very low

level, in response to a genotoxic stress (such as UV, ionizing radiation and multiple chemical DNA-damaging agents), *P53* expression is induced and exerts its functions (Gillet et al. 2000). Most of P53 activities are dependent on its ability to activate or repress transcription (Gillet et al. 2000). If DNA damage is extensive, the expression of *P53*, is induced to prevent replication of highly damaged cells (Aardema and MacGregor 2002). Thus, in the present study it appears that the induction of *P53* gene expression is most likely a consequence of the DNA damages induced by the contaminated environment. These results suggest that the exposure to uranium mining residues have the potential to induce serious genomic damages and instability, since the expression of a crucial gene, like *P53*, involved in the protection of cells against severe DNA damages and cell transformation, was significantly induced. Moreover, single nucleotide polymorphisms (SNPs) were found in the fragment of the *Rb* gene amplified for the gene expression analysis in the kidney, which is a target organ for the accumulation of metals like uranium and cadmium (Fig. 23). These polymorphisms are located in exon 11 (G to T and G to A substitutions), which results in a change of glutamate to aspartate and a valine to methionine, respectively, in the Rb protein (Fig.23). These are nonsynonymous SNPs, since the aminoacid sequence of the fragment amplified, was changed. Many genetic variations are single nucleotide polymorphisms (SNPs). Non-synonymous SNPs are “neutral” if the resulting point-mutated protein is not functionally discernible from the wild type (Bromberg and Rost 2007), however “non-neutral” non-synonymous SNPs are known to cause numerous diseases (Bromberg and Rost 2007). In this case, there are no evidences, known to the authors of this study, whether these SNPs are “neutral” or not, since there is no information on the presence and level of such polymorphisms in natural populations of rodents. Further studies are required to clarify this issue. The *Rb* gene is also a tumor suppressor gene and member of the retinoblastoma gene family (Dannenberg et al. 2004). This gene controls the G1/S transition of the cell cycle by modulating the activity of E2F transcription factors (Dannenberg et al. 2004). Hypophosphorylated pRb binds to E2Fs and forms complexes, which actively represses genes that are essential for cell cycle regulation, DNA replication, DNA repair, G2/M checkpoints and differentiation (Dannenberg et al. 2004).

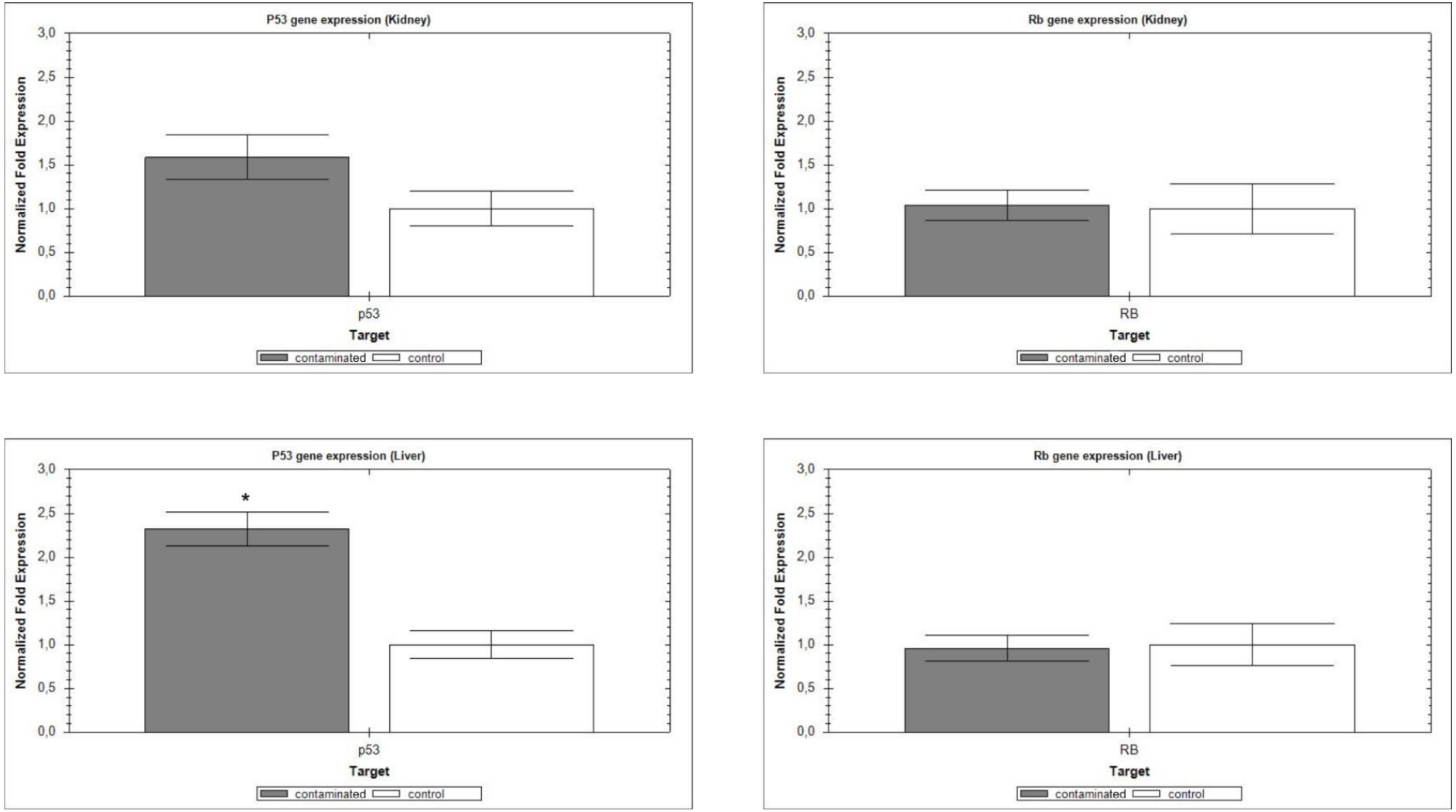

Figure 22: *Rb* and *P53* gene expression analysis in the liver and kidney of *Apodemus sylvaticus* inhabiting the reference (white) and contaminated (dark grey) sites. Data are shown as average ± standard error. (*) stands for statistical significant differences ($p < 0.05$)

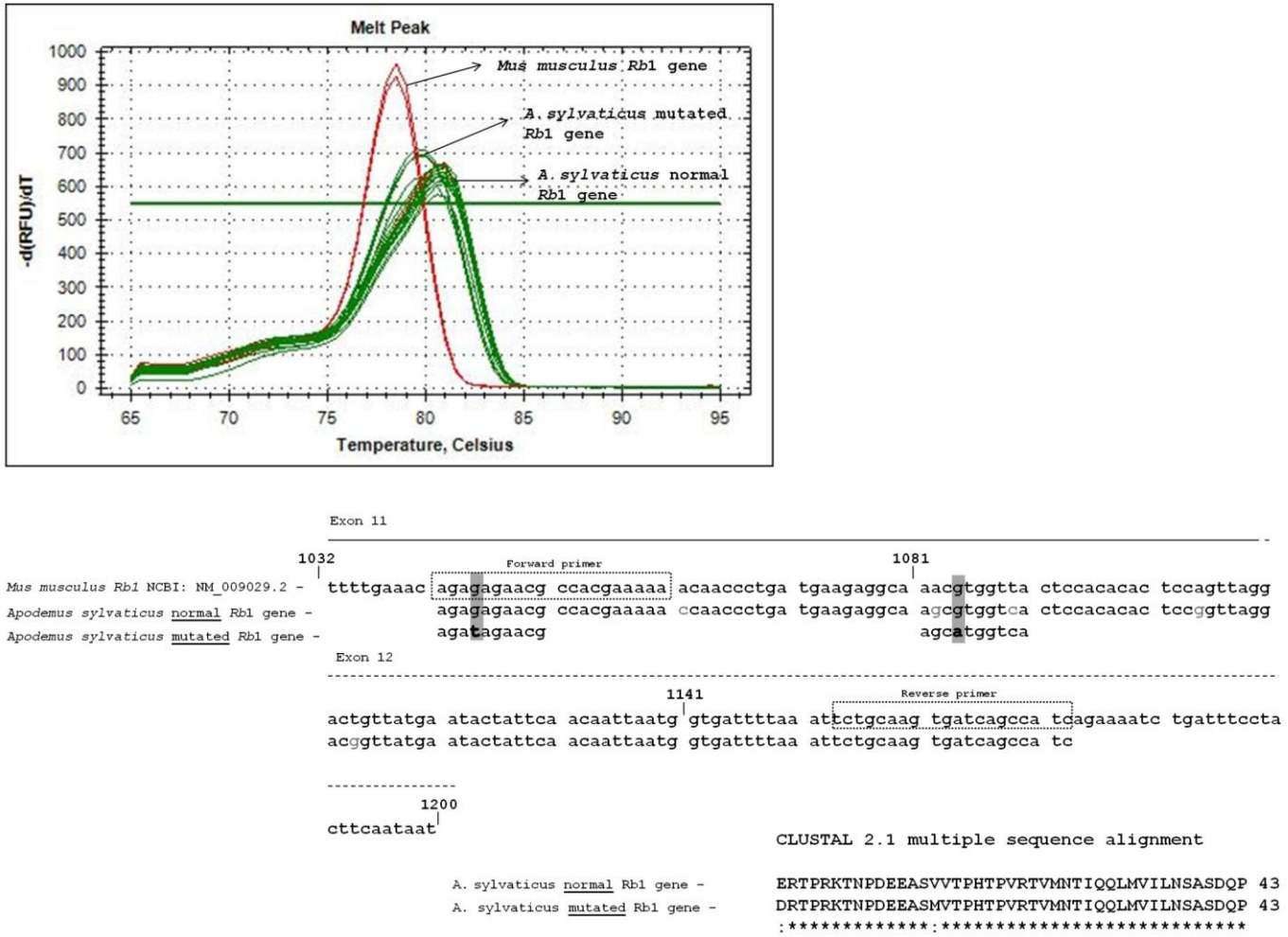

Figure 23: Single nucleotide polymorphisms in the *Rb* gene sequence amplified for gene expression analysis in the kidney samples. The image on top shows the different configuration of the melt peaks, between *Mus musculus Rb* gene sequence and the normal and polymorphic sequences of *Apodemus sylvaticus*. The image bellow shows a schematic representation of the *Rb* gene sequences from both species, and the location of the polymorphisms. Sequence alignment of both normal and polymorphic Rb proteins are shown in the bottom. This figure also show the location of the exons and primers used for the amplification.

Polymorphisms in this gene may have three outcomes: i) the polymorphisms will prove to be a result of a genetic drift, potentially improving the function of this gene and conferring the organism's better protection against tumour formation; ii) the polymorphisms were caused by exposure to uranium mining wastes and are "neutral", causing no problems in the translation process, and as so no harm to the organisms; iii) the polymorphisms were caused by exposure to uranium mining wastes and are not "neutral", potentially causing the inactivation of the Rb protein, which may result in genomic instability and cell immortality, after severe DNA damage (Manning et al. 2010). Cell immortality, aneuploidy and continuous capability for division are key factors in carcinogenesis.

6.5 Conclusions

This showed that the organisms living in the mine area bioaccumulated mainly uranium and cadmium, which may be the cause of the serious genotoxic effects observed in these organisms. These effects include DNA strand breaks and changes in the expression of important genes, like *p53,* which is crucial for the organism's defense against tumour formation and is reported to be induced in cells with extensive DNA damages. These events are a cause and a consequence of severe genomic instability and show the potential dangers of the exposure to these contaminants. Two main reasons can be highlighted for the relevance of the present study: i) it was conducted in mice, which are frequently employed as model organisms for the evaluation of toxic effects of contaminants in humans, and are therefore often used as bioindicator species for the assessment of risks of environmental exposure of humans; ii) it consists of an *in situ* evaluation of exposure to contaminants. Moreover, the presence of nonsynonymous SNPs was also reported in the fragment of the *Rb* gene amplified in this study. However, it is not clear if these polymorphisms result from a genetic drift, or if they are "neutral" or not, preventing the authors of drawing any conclusion on their origin and potential consequences.

Since small mammals are often used as bioindicator species for the assessment of human risks, the results of this study raise concerns on potential effects in human populations living nearby uranium mines, although knowing that the routes and the level of exposure of wood mice and human populations are naturally different.

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CHAPTER 7 - BIOMONITORING A POPULATION INHABITING NEARBY AN ABANDONED URANIUM MINE

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7.1 Abstract

Environmental exposure to uranium (mainly in the form of ^{238}U) and its daughter radionuclides, has been linked to several negative effects such as those related with important physiological processes, like hematopoiesis, and may also be associated with genotoxicity effects. Herein, genotoxic effects, immunotoxicity, trace elements and C reactive protein (CRP) analyses, were performed in peripheral blood samples collected from individuals of a population inhabiting nearby a deactivated uranium mine. C reactive protein analysis was performed to exclude candidates with active inflammatory processes from further evaluations. DNA damages and immunotoxicity (immunophenotyping and immune cell counts) were detected by comet assay and flow cytometry, respectively. Significant DNA damages were observed in the peripheral blood samples from the volunteers of the Cunha Baixa village. A significant decrease of NK and T lymphocytes counts were observed in the individuals from the Cunha Baixa village, when compared with individuals from the reference site. Uranium and manganese levels were significantly higher in the volunteers from the Cunha Baixa village. On the other hand, zinc levels were significantly lower in those individuals when compared with the volunteers from the control village. Results suggest that inhabitants from Cunha Baixa have a higher risk of suffering from serious diseases such as cancer, since high DNA damages were observed in peripheral blood leukocytes and also decreased levels of NK and T cells, which play an essential role in the defense against tumor growth.

Keywords: uranium wastes; ionizing radiation; genotoxicity; immunotoxicity

7.2 Introduction

Catastrophic nuclear accidents, like Chernobyl and Fukushima, attract instant attention. Tremendous resources are provided to ease the suffering of the affected populations, and also to understand/evaluate the associated health effects. However, such accidents are not the only source of radioactive hazards, since long-term exposure to uranium and radium mining wastes is also known to cause serious negative health effects in workers of uranium mines, mills and processing facilities (Schneider et al. 1999; Popp et al. 2000; Milacic and Simic 2009). Although health effects are also known to occur in populations living nearby former and active mines, there are relatively few studies focusing on that issue (Au et al. 1995; Au et al. 1998; Wagner et al. 2010; Brugge and Buchner 2011), showing a rather poor investment on the knowledge of the real problems caused by the extraction of radioactive ores. Uranium mining wastes contain uranium, radionuclides from uranium's decay chain and also carcinogenic metals (Pereira et al. 2008; Lourenço et al. 2012). After ingestion, these contaminants may exert negative effects at the molecular, cellular, tissue and organ levels. Exposure to environmental uranium has been linked with several heath consequences, and can exert toxic effects on several important physiological processes, including kidney function, bone development and hematopoiesis (Brugge et al. 2005; Wagner et al. 2010; Brugge and Buchner 2011). The bone marrow is affected by uranium, and when this element is sequestered in the bone (one of its target organs) it has the potential to alter hematopoiesis (Wagner et al. 2010). Uranium may also affect hematologic parameters indirectly by acting on the kidneys and potentially reducing erythropoietin production (Wagner et al. 2010). At a molecular level, uranium and other metals may also induce genomic instability by affecting pathways like DNA repair, regulation of nuclear transcription factors, gene expression regulation, apoptosis, cell growth, reactive oxygen species (ROS) generation and by replacing essential metals in their metabolic pathways (Wang and Shi 2001; Goyer et al. 2004; Leonard et al. 2004). All of these events may lead to the development of serious genetic diseases, like cancer.

In Portugal there are several abandoned uranium mines, located mainly in the north and center regions of the country, and a great fraction of them, are located near human populations. When uranium extraction ceased to be lucrative, the governmental company in charge, entered in liquidation (Carvalho et al. 2005a), leaving tonnes of tailings, heaps and acid/radioactive effluents without any treatment for years. This led to public exposure to uranium mining wastes, putting the health of inhabitants at risk. This was the case of the population living nearby the Cunha Baixa uranium mine, located near the city of Mangualde, in the district of Viseu, Portugal. This mine was explored from 1970 to 1993 (Neves et al. 2005). The underground and open pit works produced

about one million tonnes of wastes that were dumped in the mine area (Neves et al. 2005). From 1984 to 1993, the low grade ores produced in Cunha Baixa and adjacent mines were transferred to the open pit area and submitted to heap leaching with sulfuric acid solutions, creating an artificial pond (Antunes et al. 2008). The use of sulphuric acid as leaching agent, as well as the geologic nature of dumped materials, give rise to acidic waters (Pereira et al. 2004). These waters, which re-emerge in wells downstream, are unsuitable for human consumption, irrigation and livestock watering (Carvalho et al. 2005b; Neves et al. 2005), since these can expose the population and also breeding cattle and other domestic animals to radiation doses above the recommended limits and also to chemical toxicity from the dissolved metals (Carvalho et al. 2005b). Furthermore, the use of these waters for irrigation purposes may facilitate the transfer of radionuclides and metals throughout the food chain (Carvalho et al. 2005b). The release of radioactive and toxic contaminants (e.g. ^{222}Rn, ^{226}Ra, metals) into the air, also present hazards to residents and to the surrounding environment (Neves et al. 2005). High concentrations of several radioactive and non-radioactive chemical compounds on samples collected in different environmental compartments around the Cunha Baixa mine, were detected, indicating that the dispersion of the contaminants related with mine works, still occurs (Pereira et al. 2004; Pereira et al. 2008; Carvalho et al. 2009). The purpose of this study was to determine to what extent the exposure to the uranium mining wastes from Cunha Baixa is causing damages in the immune system of the population living in Cunha Baixa village. To that end, immune cells count and immune cells phenotype (designated immunophenotyping) in the peripheral blood, were evaluated by flow cytometry. Moreover, the presence of DNA damages in white blood cells from the peripheral blood was also investigated through DNA strand break detection using the comet assay, and the presence of trace elements was also determined in the same samples. The levels of C-reactive protein were determined to exclude from the immune cell count, all candidates with active inflammatory processes.

7.3 Materials and Methods

7.3.1 Subjects and biological samples collection

After obtaining the approval of the Administração Regional de Saúde do Centro, IP (the public healthcare administration services from the central region of Portugal), from the healthcare unit of Mangualde and from the local political authorities, the selection of the individuals that could

participate in this study, began. Firstly, the database of the population of Cunha Baixa village, registered in the healthcare unit, was analyzed and the eligible individuals were identified by their family physicians, to exclude those with drinking problems, smoking habits, autoimmune diseases and a residency period of less than 5 years (residency period was established based on a study performed by Falcão et al. 2005). After this primary selection, the eligible individuals were invited to participate in the study, through a letter sent by post mail, clearly explaining the objectives of the study. From the 70 eligible candidates, 54 agreed to participate. Interviews took place in the healthcare unit of Mangualde and upon interview, all the participants signed informed consents and relevant individual information, on age, lifestyle factors (namely smoking and alcohol consumption habits), health conditions and occupational history, were obtained by means of a questionnaire. Reference individuals were selected in another village (Vale de Açores, Mortágua) about 70 km apart from the Cunha Baixa uranium mine, but also from the district of Viseu, and with no records of any uranium extraction activity. Participants were selected using the same criteria applied to the target population of the Cunha Baixa village. After a primary selection, eligible candidates were contacted and 30 agreed to participate in the study. The demographic characteristics of both populations are described in table 17. No significant statistical differences were observed between both populations, regarding age distribution (t = -0.00681; df =82; p=0.995).

Table 17: Characteristics of the studied populations

	Control (Vale de Açores)	**Cunha Baixa**
Sample size	30	54
Age (years) (Mean ± SD)	53.90 ± 21.05	53.87 ± 14.92
Age range	17 - 75	13 - 91
Females (%)	16 (53.3)	35 (64.8)
Males (%)	14 (46.7)	19 (35.2)

Peripheral blood samples were obtained by venipuncture into vials containing K_3EDTA and also into coagulation vials with gel for serum analysis (Vacuette, Greiner Bio One, Austria), by nurses from the local healthcare units (Mangualde and Vale de Açores). A total of three 6 mL

tubes were collected from each candidate, two with K_3EDTA for DNA strand breaks, trace elements detection and immunophenotyping and one coagulation vial for the quantification of the C-reactive protein.

7.3.2 Comet assay

Comet assay was performed using 10 μl of fresh whole blood samples directly from the collection tube in 150 μl of low melting point agarose and conducted under yellow light, to prevent UV-induced DNA damage. The assay was performed with slight modifications of the protocol described by Nogueira et al. (2006): briefly, microscope slides were covered with a first agarose layer, left to dry and a second layer containing the cells was placed on top. Visual scoring of cellular DNA on each slide was based on the categorization of 100 cells randomly selected. The comet-like formations were visually graded into 5 classes, depending on DNA damage, and scored as described by Garcia et al. (2004). Positive controls were always included, and consisted of cells previously exposed to 200 μM of H_2O_2, for 1 h.

7.3.3 Immune cells count and immunophenotyping

Upon arrival, a complete blood count using the Coulter Ac Tdiff2 (Beckman Coulter, France) was performed to all the collected samples. Then, 500 μl of whole blood were washed with PBS and stained for 15 minutes in the dark, using Lymphoclonal™ (Cytognos, Salamanca, Spain) that contains the combination of the monoclonal antibodies: Lambda-FITC + CD8-FITC / Kappa-PE + CD56-PE/ CD19-PerCPCy5.5 +CD4-PerCPCy5.5 / CD3-APC to identify in a single tube a wide range of lymphoid subsets. Other monoclonal antibodies were also used in combination with Lymphoclonal™, which were CD45-Krom Orange (Beckman Coulter, France), CD5-PC7 (Beckman Coulter, France) and CD38-APC-H7 (BD Biosciences, USA). After staining, the samples were lysed using BD FACS™ Lysing Solution (BD Biosciences, USA) also for 15 minutes in the dark. After lysis, the samples were washed with PBS and analyzed on a FACSCanto™ II (BD Biosciences, Erembodegem, Belgium). During analytical experiments, 200.000 threshold events were collected per sample. The resulting files were analysed using the Infinicyt software (Cytognos, Salamanca, Spain).

The major white blood cells populations, such as eosinophils, neutrophils, monocytes, lymphocytes, basophils and dendritic cells, as well as T, B and Natural Killer (NK) lymphocytes and

also their subsets (CD4, CD8 and Tγδ for T cells; immature, naïve + memory and plasmablasts for B cells; CD56 bright and dim for NK cells), were analyzed.

The levels of C-reactive protein were determined in the Hospital Infante D. Pedro (Aveiro, Portugal), according to standard protocols. All the candidates with C-reactive protein levels above 0.5 mg L^{-1} (11 candidates from the Cunha Baixa village), were not considered for the subsequent analysis of immune cells count data.

Regarding the results of the quantification of cell populations, these were stratified according to age and sex, since there is a sexual dimorphism in the immune response (sex hormones act in the regulation of the immune response) (Bouman et al. 2005; Fish 2008), and also because in humans, the immune system undergoes changes during their life span (Mund et al. 2001). Ageing is associated with an impaired capacity of the innate immune system to produce pro- as well as anti-inflamatory cytokines, which weakens the ability to respond to infections and cancers (May et al. 2009). Therefore, results were compared between females from the contaminated and control sites included in the same age range and the same was done for males. A report from Tryphonas et al. (2001) supports this approach by stating that cell counts should be compared to normal age and sex-matched controls, for an adequate assessment of immunotoxic effects of environmental contaminants in humans. Furthermore, results on cell percentages and absolute cell counts are presented here and statistical differences were assessed for both. Absolute cell counts give the information on the number of cells present in the sample and cell percentages give the representativeness of the cell subset, regarding the main cell population where it is included.

7.3.4 Trace elements determination

Samples were digested in a closed, pressurized microwave digestion unit (Mars5, CEM) equipped with medium pressure HP500 vessels, using ultrapure HNO_3 (65%) and H_2O_2. Elements concentrations were measured in a Thermo X-series quadrupole ICP-MS (Thermo Scientific), equipped with Ni cones and a glass concentric nebulizer (Meinhard, 1.0mL min^{-1}) refrigerated with a Peltier system. The analysis of the biological samples after digestion was carried out in 5% nitric acid and ^{115}In internal standard correction. For quality control purposes the following criteria were applied: internal standard accepted variations between 70 and 130%; blank's concentrations bellow the limit of detection, elements spikes recovery between 70 and 130% and duplicates with variations lower than 20%.

7.3.5 Statistical analysis

Regarding chemical analysis and flow cytometry results the statistical analysis was performed using one-way ANOVAs and Kruskal-Wallis one-way analysis of variance on ranks, whenever the data failed to meet the normality and homoscedascity assumptions. Comet assay results were analyzed using one-way ANOVA. The level of significance defined for all the analyses was 0.05.

7.4 Results and Discussion

Trace elements analyses in the peripheral blood revealed that Mn and U were present in significantly higher levels in individuals from the Cunha Baixa village, when compared to those from the control village (Vale de Açores) (F = 29.3; df = 1, 39; $p < 0.001$ for Mn; H = 5.86; df = 1; p = 0.015 for U) (Table 18). This was likely caused by the exposure to the uranium mining wastes widespread near the Cunha Baixa village, since these wastes are heavily contaminated by these metals (Pereira et al. 2008; André et al. 2009; Lourenço et al. 2012). The population of the Cunha Baixa village may be exposed to these contaminants either through inhalation of dusts or through the ingestion of contaminated food or water (Carvalho et al. 2005b; Neves et al. 2005; Pereira et al. 2009), since the information collected from the questionnaires revealed that the majority of the population eat local products and drink water from local sources, other than the public water supply. Considering zinc, control individuals had significant higher levels of Zn (F = 11.9; df = 1, 39; p = 0.001), when compared with individuals from the Cunha Baixa village (Table 18). The reasons behind the decrease of this essential trace element in whole blood samples of the individuals from the Cunha Baixa village are yet to be clarified. A study performed by Iyengar et al. (1988), was able to propose reference values for several elements, including Mn and Zn, but not for U, in human whole blood. According to this study, the levels of Zn in whole blood of individuals from both the reference area and the Cunha Baixa village were within the reference ranges, however for the individuals from the Cunha Baixa village it was close to the lower limit of the reference range. On the other hand, the level of manganese, also an essential trace element, was also within the normal range of values but very close to the upper limit of the reference range for the same individuals.

Table 18: Metal content of whole blood samples of individuals from the control and contaminated sites (μg.L-1) (mean ± standard deviation)

Sampling sites	**Be**	**Mn**	**Ni**	**Cu**	**Zn**	**Sr**	**Cd**	**Pb**	**U**
Whole blood metal content ± SD (μg.L^{-1})									
Control Site	UDL	9.1 ± 2.6	UDL	830.3 ± 193.7	7103.9 ± 1192.4	17.3 ± 5.1	UDL	32.2 ± 22.8	0.07 ± 0.02
Cunha Baixa	UDL	15.2 ± 3.3(*)	UDL	985.5 ± 347.2	5579.2 ± 1218.4(*)	18.1 ± 5	UDL	35.7 ± 26.8	0.09 ± 0.03(*)

(*) Statistical significant differences between sampling sites, p <0.05.
UDL: Under Detection Limits

To evaluate the potential genotoxic effects caused by the exposure to uranium mining wastes, whole blood samples were also analyzed by the comet assay. This technique is used to detect the presence of DNA strand breaks, which is considered a sensitive indicator of genotoxicity (Shugart 2000). Results showed that there was a significant loss of DNA integrity in individuals from the Cunha Baixa village, aged between 40 and 60 years and older than 60 years (F = 11.4; df = 1, 30; p < 0.001 and F = 36.3; df = 1, 35; p < 0.001, respectively) (Fig 24).

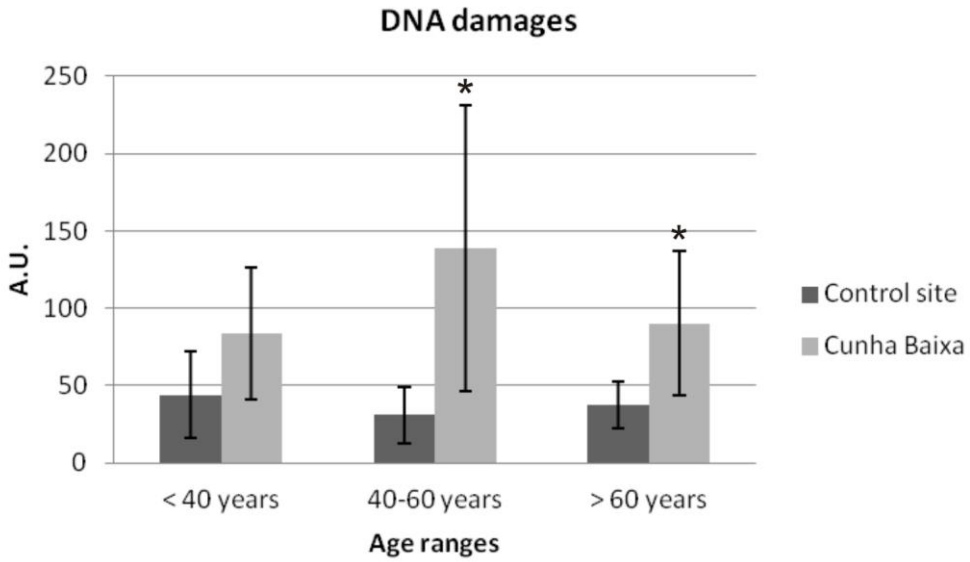

Figure 24: Whole blood DNA integrity in the population inhabiting the reference (light grey) and contaminated (dark grey) sites. Data are shown as average ± standard deviation. (*) stands for statistical significant differences (p <0.05)

These observations suggest that this loss of DNA integrity might have been caused by the exposure to uranium and its daughter radionuclides, and also to metals like manganese, that were present in the mining wastes and detected in the blood of exposed individuals at higher levels than in the individuals from the reference village. Manganese is known to cause damage on the integrity of the DNA chain in human peripheral blood lymphocytes (Gerber et al. 2002), probably through the induction of oxidative DNA damage (Jameton et al. 2002). Moreover, the fidelity of DNA replication decreases substantially in the presence of Mn^{2+} by modifying the activity of DNA polymerase (Gerber et al. 2002), which can give rise to DNA damage in the form of mutations. The exposure to uranium and its daughter radionuclides, is also known to cause severe DNA damages. Due to its relatively low radioactivity, uranium exerts its toxic effects on the DNA molecule, mainly

chemically, through the production and accumulation of reactive oxygen species (ROS) inside the cells, causing several types of lesions like DNA strand breaks, DNA-protein crosslinks, alkali labile sites and oxidative base modifications (Barillet et al. 2010). As for uranium daughter radionuclides present in the mining wastes of Cunha Baixa (Carvalho et al. 2007), DNA damages are inflicted mainly through the emission of alpha radiation. The ingestion or inhalation of alpha emitting radionuclides increases the risk of severe damage to the cells and consequently cancer, due to the emission of high Linear Energy Transfer (LET) radiation (UNSCEAR 2000, 2006a). High LET radiation (neutrons, alpha particles, with high ionization capacity) is able to cause clustered DNA damages, which consists in closely spaced DNA lesions of the same or different nature, that consequently are complex and difficult to repair (Hada and Georgakilas 2008). As a result, they have been reported to have a high mutagenic potential (Hada and Georgakilas 2008), potentially giving rise to chromosomal aberrations leading to severe alterations of the genetic material. Cytotoxic effects of ionizing radiation are thought to result mainly from incompletely or incorrectly repaired DNA lesions (Hada and Georgakilas, 2008). The fact that DNA damages were significantly higher in the older individuals, may be related to the age-associated accumulation of DNA damage, which is attributable to an age-related increase in ROS production and a decline in repair capacity (Chen et al., 2007). Such decline has been observed in *in vitro* and *in vivo* systems (Chen et al., 2007). However, according to this hypothesis, higher level of DNA damages would be expected in individuals older than 60 years. Another possible explanation may be related to the accumulation and decay of radionuclides in the bone. Radium is such an example, since it deposits in the bone due to its similarity with calcium, within areas where new bone mineral is being formed and also on all bone surfaces (USATSDR and USEPA, 1990). Radium will remain in the areas of new bone formation, but the radium deposits on bone surfaces will eventually move into compact bone while new bone matrix is deposited on top (USATSDR and USEPA, 1990). In these deposition processes, ^{226}Ra will remain in the skeleton indefinitely (USATSDR and USEPA, 1990) and will start to decay, forming new and more radioactive products like ^{222}Rn and ^{210}Po that will increase damages to the cells that are being formed in the hematopoiesis process. It can also be suggested that the damages detected in individuals older than 60 years are lower than those detected in individuals with ages between 40 and 60 years, probably because a decreased rate of cell renewal is normally observed during the aging process.

The immune system is one of the most complex systems of the human body. It is composed of several cell types (lymphocytes, granulocytes, monocytes), perfectly adapted to recognize, neutralize and destroy non-self or foreign substances and cells, protecting against infection and

cancer (UNSCEAR 2006a). It has been known that metals, especially uranium and ionizing radiation, have negative effects on the hematopoietic system and have the ability to modulate the immune response in a variety of animal species including humans (Zelikoff 1998; Kiang et al. 2010; Bao et al. 2012). One way of estimating those effects is by estimating changes in cells phenotype and cell numbers (UNSCEAR 2006a). Herein those changes were evaluated by flow cytometry, a technique that is used for the identification and quantification of cell populations and also to recognize phenotypic abnormalities, even when these are present in a small proportion of the cells analyzed (Craig and Foon 2008). Results revealed no phenotype abnormalities in none of the cell populations. However, differences were found, regarding immune cell counts, in some of the cell populations analyzed. Regarding females, significant differences were observed in total white blood cell (WBC) count (F = 6.3; df = 1, 13; p = 0.027), between individuals with ages bellow 40 years from the control and contaminated sites, showing an increase in WBC in females from the Cunha Baixa village (Table 19).

The same was observed for neutrophils (F = 5.3; df = 1, 13; p=0.04) in the same individuals (Table 19), suggesting that the increase in WBC may result from the increase in neutrophils absolute counts. A study conducted by Lorimore and co-authors (2001) in mice, showed that hematopoietic tissues exposed to ionizing radiation exhibited increased macrophage activation, which was associated with an unexpected neutrophil infiltration into the spleen. These authors also showed that macrophage activation and neutrophil infiltration were not direct effects of irradiation, but a consequence of the recognition and clearance of radiation-induced apoptotic cells. Radiation exposure may also cause cells to undergo necrosis, which would also trigger inflammatory-type responses (Hatfield et al. 2005). Moreover, in a study performed by Wagner et al. (2010), on a community living near an uranium processing mill, it was also observed that exposure to uranium has increased neutrophils counts. A significant increase of plasmablast absolute counts (F = 44.8; df = 1, 13; p < 0.001) and percentage (F = 12.1; df = 1, 13; p = 0.005) (Table 19), was observed in women aged bellow 40 years from the Cunha Baixa village. The representativeness of these cells also increased significantly in men of the same age (F = 40.7; df = 1, 10; p = 0.039) but decreased in men older than 60 years (F = 17.4; df = 1, 9; p = 0.003) (Table 19). No information was found linking changes of plasmablast cell percentages and absolute counts and exposure to metals and/or radionuclides. Further studies are required to clarify this issue. A significant decrease in basophils representativeness regarding the total white blood cell population was observed in women aged bellow 40 years (F = 7.7; df = 1, 10; p < 0.02), from the Cunha Baixa village (Table 19). Since no significant differences were observed in basophils

absolute counts, this was probably caused by the increase of neutrophils counts which in turn increased the total white blood cell counts, thus reducing the representativeness of basophils in those individuals. A significant increase of circulating dendritic cells absolute counts and percentage was observed for men aged bellow 40 (F = 14.9; df = 1, 8; p = 0.018 and F = 10.2; df = 1, 10; p = 0.049, respectively). Moreover, it was also observed a decrease of circulating dendritic cells absolute counts in women older than 60 years (F = 5.7; df = 1, 14; p = 0.033) from the Cunha Baixa village (Table 19). Some studies cited in a review published by Hatfield et al. (2005), report that apoptosis triggered by ionizing radiation, may induce the recruitment of dendritic cells to the affected sites, which can be the cause of their increase in the peripheral blood. However, the biological processes involved in the response of dendritic cells to ionizing radiation are not yet fully understood. On the other hand, no explanation was found for the significant decrease of dendritic cells observed in women older than 60 years. However, it may be related to advanced age, since their absolute number in peripheral blood samples progressively decline with increasing age (Della Bella et al., 2007). A significant decrease in lymphocytes absolute counts and representativeness was observed in men aged bellow 40 living in the Cunha Baixa village (F = 11.2; df = 1, 10; p = 0.03 and F = 12.5; df = 1, 10; p = 0.04, respectively) (Table 19). This was probably due to the significant decrease of natural killer (NK) cells absolute counts and percentages also observed in these individuals (F = 10.8; df = 1, 10; p = 0.03 and F = 18.5; df = 1, 10; p = 0.015, respectively) (Table 19). A significant decrease in lymphocytes representativeness was also observed in women aged bellow 40 living in the Cunha Baixa uranium mine (F = 12.1; df = 1, 13; p=0.005) (Table 19). A decrease of natural killer (NK) cells absolute counts and percentages was also observed in women from Cunha Baixa and aged bellow 40 years (F = 7.3; df = 1, 13; p = 0.019 and F = 12.1; df = 1, 13; p = 0.005, respectively) (Table 19), however this decrease was not the cause for the decrease of lymphocytes representativeness, since there was no significant differences in absolute counts between individuals from the Cunha Baixa village and from the reference site. This was probably due to the increase of total white blood cells in these individuals, which was already discussed here. A significant decrease in NK $CD56^{dim}$ absolute counts was also observed in women and men aged bellow 40 years (F = 6.01; df = 1, 13; p=0.03 and F = 11.2; df = 1, 10; p = 0.029, respectively) from the same population (Table 20). Also, a significant decrease was observed in NK $CD56^{bright}$ absolute cell counts in women aged bellow 40 years and older than 60 years (H = 6.2; df = 1; p=0.011 and F = 10.2; df = 1, 14; p=0.007, respectively) (Table 20).

Table 19: Quantification of main WBC (mean ± standard deviation of percentages and absolute cell counts) populations in individuals from the control and contaminated sites.

Control	Men						Women					
	<40		40-60		>60		<40		40-60		>60	
	%	(cells/µl)	%	(cells/µl)	%	(cells/µl)	%	(cells/µl)	%	(cells/µl)	%	(cells/µl)
WBC count		7833 ± 971		9000 ± 2546		6833 ± 839		7129 ± 1266		6900 ± 930		8175 ± 1353
Eosinophils	2.9 ± 2	217 ± 137	2 ± 1	170 ± 35	4.7 ± 2.2	307 ± 119	1.9 ± 2.1	144 ± 170	2.7 ± 1.9	188 ± 132	3.5 ± 1.7	304 ± 189
Neutrophils	50.3 ± 13.9	4026 ± 1591	68.1 ± 12.8	6290 ± 2881	65.6 ± 8.2	4525 ± 1122	58.6 ± 9.3	4211 ± 1123	57.8 ± 6.1	3961 ± 443	61.9 ± 9	5009 ± 826
Monocytes	6.2 ± 0.9	481 ± 25	6.6 ± 2	570 ± 9	5.5 ± 2.1	389 ± 202	5.4 ± 1.1	396 ± 148	4.7 ± 0.5	323 ± 55	5.4 ± 1	439 ± 89
Lymphocytes	33.9 ± 4.7	2641 ± 319	21.8 ± 9.6	1841 ± 314	21.5 ± 7.4	1432 ± 373	34.8 ± 7.2	2463 ± 571	32.9 ± 5	2290 ± 593	25.8 ± 8.5	2160 ± 973
Plasmablasts	0.003 ± 0.003	0.2 ± 0.1	0.01 ± 0.002	1 ± 0.3	0.02 ± 0.01	1 ± 0.1	0.003 ± 0.003	0.5 ± 0.5	0.01 ± 0.003	0.3 ± 0.5	0.02 ± 0.01	2 ± 1
Basophils	0.6 ± 0.4	50 ± 27	0.4 ± 0.2	29 ± 10	0.4 ± 0.1	30 ± 7	0.6 ± 0.3	38 ± 15	0.9 ± 0.2	70 ± 25	0.5 ± 0.2	38 ± 20
Dendritic cells	0.73 ± 0.3	58 ± 27	1.1 ± 0.1	96 ± 33	1.7 ± 0.2	116 ± 9	0.77 ± 0.5	58 ± 49	1.3 ± 0.4	95 ± 37	2 ± 1.4	157 ± 84
T Lymphocytes	23.9 ± 5.1	1843 ± 259	15 ± 9.6	1232 ± 480	15.7 ± 6.5	1040 ± 347	24.4 ± 9	1720 ± 632	22.8 ± 3.4	1565 ± 276	18.9 ± 6.3	1564 ± 676
B Lymphocytes	4.1 ± 1.5	329 ± 155	2.1 ± 0.8	199 ± 125	1.4 ± 0.6	89 ± 35	2.7 ± 1.6	195 ± 155	4.6 ± 3.9	347 ± 339	2.9 ± 1.8	252 ± 191
NK cells	8.3 ± 1	670 ± 195	3.3 ± 1.3	279 ± 31	4.4 ± 0.4	297 ± 19	7.2 ± 2.6	519 ± 216	3.6 ± 1.9	256 ± 138	4.2 ± 1	350 ± 132
Cunha Baixa												
WBC count		7050 ± 2475		8425 ± 1220		7429 ± 955		9171 ± 1741(*)		7750 ± 1540		8364 ± 3708
Eosinophils	5.5 ± 5.6	320 ± 261	3.3 ± 2.5	271 ± 178	3.5 ± 2.3	264 ± 185	2.7 ± 1.2	240 ± 90	2.84 ± 1.2	226 ± 116	3 ± 1.2	268 ± 204
Neutrophils	64.1 ± 2.6	4554 ± 1770	61.4 ± 6	5212 ± 1105	61.8 ± 6.7	4603 ± 863	65.3 ± 8.7	6085 ± 1841(*)	60.6 ± 7.8	4732 ± 1246	63.9 ± 9.5	5614 ± 3688
Monocytes	6.9 ± 1.2	472 ± 82	6.5 ± 1.6	547 ± 151	5.2 ± 1.5	375 ± 70	5.5 ± 0.9	504 ± 152	4.76 ± 0.9	372 ± 120	5 ± 1.8	377 ± 113
Lymphocytes	19.8 ± 3.7(*)	1443 ± 750(*)	22.9 ± 6.1	1925 ± 537	19.8 ± 5.7	1464 ± 444	21.7 ± 6.8(*)	1922 ± 421	27.6 ± 7.5	2098 ± 533	22.7 ± 7.6	1712 ± 563
Plasmablasts	0.03 ± 0.01(*)	2 ± 1	0.04 ± 0.05	4 ± 4	0.005 ± 0.003(*)	0.5 ± 0.3	0.02 ± 0.01(*)	2 ± 1(*)	0.03 ± 0.04	3 ± 4	0.02 ± 0.01	2 ± 1
Basophils	0.3 ± 0.2	21 ± 4	0.5 ± 0.2	41 ± 16	0.5 ± 0.2	39 ± 15	0.4 ± 0.3(*)	34 ± 19	0.6 ± 0.2	43 ± 18	0.3 ± 0.2	21 ± 13
Dendritic cells	1.8 ± 0.5(*)	119 ± 8(*)	1.3 ± 0.6	107 ± 36	1.2 ± 0.6	89 ± 48	1.2 ± 0.4	110 ± 60	1 ± 0.3	75 ± 16	1.1 ± 0.6	83 ± 39(*)
T Lymphocytes	16.3 ± 4.9	1207 ± 750	19 ± 5.4	1566 ± 477	18.7 ± 5.9	1395 ± 523	18.5 ± 5.2	1643 ± 307	24 ± 6.1	1826 ± 432	19.6 ± 6.4	1467 ± 450
B Lymphocytes	3.6 ± 1.2	236 ± 1	3.7 ± 1.6	307 ± 132	1.9 ± 1	136 ± 57	3.2 ± 2	278 ± 155	3.5 ± 1.7	268 ± 120	2.8 ± 1.5	212 ± 97
NK cells	1.5 ± 1.3(*)	119 ± 128(*)	4.5 ± 2.2	412 ± 146	4.3 ± 1.4	307 ± 67	3.2 ± 1.7(*)	273 ± 107(*)	3.1 ± 1.2	270 ± 112	4.3 ± 2.3	317 ± 128

(*)stands for statistical significant differences between sampling sites, p <0.05.

Table 20: Quantification of Natural Killer cells (NK cells) subsets (mean ± standard deviation of percentages and absolute cell counts) in individuals from the control and contaminated sites.

	NK cells											
	Men						Women					
	<40		40-60		>60		<40		40-60		>60	
Control	**%**	**cells/µl**	**%**	**cells /µl**	**%**	**cells /µl**	**%**	**cells /µl**	**%**	**cells/µl**	**%**	**cells/µl**
CD56^{dim}	92.9 ± 5.2	636 ± 161	98 ± 0.1	273 ± 30	95.8 ± 2.8	285 ± 26	92.2 ± 5.2	482 ± 211	93.5 ± 2.3	241 ± 136	94.6 ± 1.4	331 ± 124
CD56^{bright}	7.1 ± 5.2	34 ± 34	2 ± 0.1	6 ± 0.3	4.2 ± 2.8	12 ± 7	7.8 ± 5.2	37 ± 34	6.5 ± 2.3	15 ± 5	5.5 ± 1.4	20 ± 11
Cunha baixa												
CD56^{dim}	90.2 ± 1.6	108 ± 118$^{(*)}$	97.9 ± 1.1	362 ± 146	98.3 ± 1	302 ± 66	95.3 ± 3.1	262 ± 107$^{(*)}$	95.5 ± 5.3	231 ± 102	96.8 ± 2.6	309 ± 129
CD56^{bright}	9.8 ± 1.6	11 ± 11	2.1 ± 1.1	7 ± 2	1.7 ± 1	5 ± 4	4.7 ± 3.1	11 ± 6$^{(*)}$	4.5 ± 5.3	13 ± 21	3.2 ± 2.6	8 ± 4$^{(*)}$

(*) Statistical significant differences between sampling sites, p <0.05.

The overall decrease of NK cells may be related to the decrease of zinc levels, observed in the individuals from the Cunha Baixa village, since this element is involved in the development of NK cells from $CD34^+$ cell progenitors (John et al. 2010) and is very important for the maintenance of NK cell function (Ertekin et al. 2004; John et al. 2010), by influencing their immunocompetence (Ertekin et al. 2004). Currently, there is no doubt that zinc is important, not only for NK cells, but also for the entire immune system (Ertekin et al. 2004). Ionizing radiation also has a negative impact on NK cells, since in a study performed by Gridley et al. (2002b), it was observed that mice irradiated with high LET radiation suffered a decrease in NK cell numbers. The overall decrease of NK cells may have serious consequences, since these cells are an important defense against viral infections and are the major cell type involved in immune surveillance against neoplastic cells (Gridley et al. 2002a; Gridley et al. 2002b). Human NK cells can be subdivided into different populations based on the relative expression of the surface marker CD56, which can be $CD56^{bright}$ (responsible for immunoregulatory cytokine production) or $CD56^{dim}$ (cytotoxic) (Poli et al. 2009). The differences observed in NK cells with $CD56^{dim}$ phenotype may additionally be explained by the reactive oxygen species (ROS) production in the bone marrow, due to the exposure to metals like uranium and ionizing radiation, since these cells are known to be sensitive to ROS production due to their lower antioxidant capacity (Harlin et al. 2007). No specific explanation was found for the significant decrease of NK $CD56^{bright}$ cells in women aged bellow 40 and older than 60 years. Further studies are needed to clarify this observation. No significant differences were found in the representativeness of each lymphocyte subset within the total lymphocyte population (Table 21). A significant decrease was also observed in $CD4^+$ T cells absolute counts in men aged bellow 40 years (F = 12.4; df = 1, 10; p=0.02) from the Cunha Baixa village (Table 22), as well as a significant decrease in $CD4^+CD56^-$ and $CD8^+$ $CD56^-$ T cells absolute counts (F = 12.1; df = 1, 10; p=0.025 and F = 8.4; df = 1, 10; p=0.04) (Table 22). The cell surface immunoglobulin superfamily molecule, CD56, is expressed only by a subset of human T lymphocytes (Kelly-Rogers et al. 2006), being typically expressed by natural killer cells, neurons , and some tumour cells (Kelly-Rogers et al. 2006). CD56 T cells are heterogeneous in nature and include $CD4^+$ and $CD8^+$ cells, among others (Kelly-Rogers et al. 2006). Functionally, $CD56^+$ T cells display properties of both NK cells and T cells (Kelly-Rogers et al. 2006). Zinc deficiency adversely affects lymphocyte proliferation (John et al. 2010). Regeneration of CD4+ T lymphocytes and $CD8^+$ $CD73^+$ $CD11b^-$ precursors of cytolytic T cells, are decreased in zinc-deficient subjects with impaired immune function (John et al. 2010), thus probably explaining the T lymphocyte decrease in men from the Cunha Baixa village.

Table 21: Proportion of lymphocytes subpopulations (mean ± standard deviation of percentages) within the total lymphocyte population.

	Lymphocytes subpopulations (%)					
	Men			Women		
Control	**<40**	**40-60**	**>60**	**<40**	**40-60**	**>60**
T Lymphocytes	60.5 ± 7	65.6 ± 6.1	71.6 ± 6	68.7 ± 11.3	70 ± 11.2	73.5 ± 10.5
B Lymphocytes	16.8 ± 0.2	11.7 ± 8.8	6.1 ± 1	8.1 ± 5	16.7 ± 8.3	10.7 ± 3.8
NK cells	11 ± 9.4	11.6 ± 8.8	22.4 ± 6.9	23.2 ± 8.5	13.3 ± 9.6	15.8 ± 7.8
Cunha baixa						
T Lymphocytes	75.7 ± 5.5	69.1 ± 7.2	74.4 ± 7.9	75.5 ± 5	76.6 ± 5.9	72.2 ± 7.7
B Lymphocytes	18 ± 17.8	12.3 ± 3.4	7.8 ± 3.4	8.9 ± 3.4	11.1 ± 2.8	10.5 ± 4.5
NK cells	6.3 ± 4.6	18.2 ± 8.5	17.3 ± 9	15.6 ± 4.3	10.3 ± 4.4	17.9 ± 5.5

Table 22: Quantification of T lymphocytes subsets (mean ± standard deviation of percentages and absolute cell counts) in individuals from the control and contaminated sites.

	T Lymphocytes											
	Men						Women					
	<40		40-60		>60		<40		40-60		>60	
Control	**%**	**(cells/µl)**	**%**	**(cells/µl)**	**%**	**(cells/µl)**	**%**	**(cells/µl)**	**%**	**(cells/µl)**	**%**	**(cells/µl)**
$CD4^+$	59.7 ± 4.9	1093 ± 81	69.4 ± 9.4	878 ± 449	61.1 ± 5.8	635 ± 229	60.5± 6.6	1051 ± 459	66 ± 10.9	1052 ± 341	62 ± 6.7	960 ± 395
$CD56^+$	1.2 ± 1.4	13 ± 16	1.3 ± 1.5	15 ± 19	3 ± 1.9	21 ± 17	1 ± 1.4	9 ± 13	14.1 ± 24.4	144 ± 229	1.7 ± 0.7	16 ± 9
$CD56^-$	98.8 ± 1.4	1080 ± 74	98.7 ± 1.5	863 ± 430	97 ± 1.9	614 ± 213	99 ± 1.4	1042 ± 460	85.9 ± 24.4	908 ± 412	98.3 ± 0.7	944 ± 388
$CD8^+$	36.7 ± 3.2	682 ± 155	26.1 ± 5.1	310 ± 63	32.7 ± 9.4	342 ± 144	31.2 ± 7.5	529 ± 178	28.3 ± 11.1	421 ± 107	32.5 ± 5.1	510 ± 228
$CD56^-$	94.6 ± 1.8	645 ± 149	91.9 ± 2.1	285± 65	74.7 ± 7.9	249 ± 93	90.8 ± 4.5	477 ± 151	77.3 ± 18.4	311 ± 42	86.6 ± 3.5	439 ± 193
$CD56^+$	5.4 ± 1.8	37 ± 13	8.1 ± 2.1	24 ± 1	25.3 ± 7.9	93 ± 54	9.2 ± 4.5	52 ± 35	22.7 ± 18.4	110 ± 112	13.4 ± 3.5	71 ± 38
Ratio $CD4^+/CD8^+$	1.6 ± 0.3		2.7 ± 0.9		2 ± 0.6		2.1 ± 0.7		2.8 ± 1.5		2 ± 0.6	
Tγδ	6.7 ± 6.8	124 ± 120	4.4 ± 4.4	44 ± 33	5.3 ± 3.8	53 ± 43	7.1 ± 2.1	124 ± 65	5.7 ± 3.7	91 ± 68	2.4 ± 2.1	39 ± 35
Cunha baixa												
$CD4^+$	56 ± 10.4	636 ± 295(*)	63.6 ± 5.7	1010 ± 320	64.4 ± 10	896 ± 354	57.4 ± 6.7	943 ± 185	66.1 ± 7.9	1227 ± 398	69.5 ± 10.6	1037 ± 395
$CD56^+$	1.2 ± 1	6 ± 3	1.6 ± 1.4	12 ± 5	1.6 ± 2.1	15 ± 23	1.2 ± 0.6	11 ± 5	3.2 ± 4.5	45 ± 91	2.5 ± 1.7	24 ± 17
$CD56^-$	98.8 ± 1	631 ± 298(*)	98.4 ± 1.4	998 ± 366	98.4 ± 2.1	881 ± 348	98.8 ± 0.6	931 ± 185	96.8 ± 5.4	1182 ± 373	97.5 ± 1.7	1013 ± 393
$CD8^+$	28.5 ± 6.4	367.9 ± 291	31.9 ± 5.4	487 ± 130	32.9 ± 10.1	464 ± 255	33.9 ± 4.9	556 ± 121	29.3 ± 7.1	520 ± 110	26.2 ± 9.8	359 ± 120
$CD56^-$	87.4 ± 14.6	300 ± 201(*)	85.7 ± 16.6	420 ± 136	85.4 ± 9.4	402 ± 248	85.6 ± 8.1	476 ± 8	86.1 ± 10	447 ± 108(*)	83.6 ± 10.6	295 ± 121
$CD56^+$	12.6 ± 14.6	68 ± 90	14.3 ± 16.6	67 ± 72	14.6 ± 9.4	62 ± 40	14.4 ± 8.1	80 ± 46	13.9 ± 10	73 ± 58	16.4 ± 10.6	54 ± 27
Ratio $CD4^+/CD8^+$	1.7 ± 0.4		2.1 ± 0.5		3.3 ± 1.1		2.1 ± 0.8		2.4 ± 0.9		3.1 ± 1.8	
Tγδ	15 ± 3.9	196 ± 160	4.2 ± 3.1	66 ± 56	2.2 ± 1.2	28 ± 15	8.2 ± 2.8	139 ± 64	4 ± 4.3	68 ± 60	3.8 ± 5.4	65 ± 102

(*) Statistical significant differences between sampling sites, $p < 0.05$.

Moreover, a report from the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR 2006b), refers that regarding lymphoid cell composition and function, the main type of damage observed in individuals exposed to ionizing radiation is the impairment of T-cell immunity, especially owing to a decreased proportion of $CD4^+$ helper, $CD4^+$ naïve and $CD8^+$ cytotoxic T-cells. Also, Wagner et al. (2010) reported a decrease in lymphocytes count in a community living near an uranium processing mill. T Lymphocytes are also considered key cells of the immune system for tumor surveillance (Igney and Krammer 2002), which means that a significant decrease of the levels of these cells may render the individual more susceptible for cancer development. A significant increase in $CD8^+CD56^-$ cells absolute counts (F = 7.1; df = 1, 12; p=0.02), was observed in women between 40-60 years from the Cunha Baixa village (Table 22). No explanation was found for this observation, evidencing the need of further studies. Regarding the B cell compartment, there is a significant decrease in the representativeness of immature B cells in men older than 60 years living in the Cunha Baixa village (F = 8.3; df = 1, 9; p=0.02), which lead to the significant increase in the representativeness of memory + naïve B cells (F = 9.8; df = 1, 9; p=0.01) in the same individuals (Table 23). In a study performed by Caraux et al. (2010) no correlation was found between age and percentages or absolute counts of total circulating B cells, immature B lymphocytes or naïve B lymphocytes, as so the significant decrease of immature B cell representativeness may be a sign of hematopoiesis disturbance due to the exposure to high LET radiation and to metals, since they are known to negatively affect this process (Wagner et al. 2010).

Table 23: Quantification of B lymphocytes subsets (mean ± standard deviation of percentages and absolute cell counts) in individuals from the control and contaminated sites.

	B Lymphocytes											
	Men						Women					
	<40		40-60		>60		<40		40-60		>60	
Control	**%**	**(cells/μl)**	**%**	**(cells/μl)**	**%**	**(cells/μl)**	**%**	**(cells/μl)**	**%**	**(cells/μl)**	**%**	**(cells/μl)**
Imature	3.6 ± 0.8	12 ± 8	8.5 ± 1.8	18 ± 14	13.8 ± 9.1	14 ± 13	5.1 ± 2	9 ± 4	7 ± 3.3	28 ± 35	7.7 ± 4.3	16 ± 11
Naive + memory	95.1 ± 0.9	312 ± 145	91.2 ± 1.6	181 ± 111	85 ± 9.1	75 ± 25	92.7 ± 2.8	183 ± 112	92.2 ± 2.9	317 ± 306	91.6 ± 4.3	234 ± 189
Plasmablasts	0.5 ± 0.2	2 ± 1	0.3 ± 0.2	1 ± 0.2	1.1 ± 0.02	1 ± 0.2	2.2 ± 1.9	3 ± 3	0.8 ± 0.8	1 ± 1	0.7 ± 0.4	1 ± 0.3
Cunha Baixa												
Imature	9.3 ± 7	22 ± 17	9 ± 4.9	26 ± 12	6.7 ± 3.2(*)	6 ± 5	7.2 ± 2.8	23 ± 18	5.4 ± 2.1	15 ± 7	6.7 ± 3.2	16 ± 10
Naive + memory	89.5 ± 6.1	211 ± 15	90 ± 5.1	278 ± 126	95.3 ± 1.6(*)	129 ± 53	91.4 ± 2.2	253 ± 139	93.5 ± 2	251 ± 113	92.1 ± 3.3	194 ± 89
Plasmablasts	1.2 ± 1	3 ± 2	1 ± 1.1	3 ± 3	0.6 ± 0.5	1 ± 0.4	1.3 ± 1	3 ± 1	1.1 ± 0.9	4 ± 4	1.2 ± 1.9	2 ± 3

(*) Statistical significant differences between sampling sites, p <0.05.

7.5 Conclusions

This study showed that the exposure to mining wastes from the Cunha Baixa uranium mine has negative effects on the community inhabiting the Cunha Baixa village. These include genotoxic effects, accumulation of metals like manganese and uranium, decrease in zinc levels and decrease of important cellular compartments of the immune system such as NK and T lymphocytes. The DNA damages found in white blood cells from peripheral blood samples suggest damages in hematopoietic organs, such as the bone marrow and for instance the thymus, where the production and differentiation of immune cells is performed. This was also evidenced by decreased numbers of NK cells and T lymphocytes, observed in Cunha Baixa volunteers, mainly in those aged bellow 40 years, which may indicate a greater susceptibility the immune system of young individuals to the effects of ionizing radiation, uranium and decreased zinc levels. Decreased levels of NK and T lymphocytes, may have serious consequences since it increases the susceptibility to infection and most importantly to cancer, because these cells are a very important line of surveillance and defense against neoplastic growth (Gridley et al. 2002a). In this scenario, a decreased defense against cancer development is a problem of great concern, considering the high risks of carcinogenesis potentiated by the exposure to radioactive wastes. Therefore, immediate intervention measures are needed to minimize the consequences and reduce the exposure of the population to the mining wastes.

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CHAPTER 8 – SINOPSIS AND FUTURE PERSPECTIVES

8.1 Synopsis

The end of the cold war in the late 1980's, was characterized by conferences on disarmament and subsequently by less or no future demand of nuclear weapons, and dilution of highly enriched uranium with depleted uranium (Merkel and Schipek 2011). This latter initiative resulted in a severe drop of uranium prices worldwide, and consequently the closure of many uranium mines, uranium processing facilities and all plants for the recovery of uranium from phosphate ore (Merkel and Schipek 2011). However, in 2004 the uranium price started to rise, caused by a continuous request of nuclear fuel and construction of new nuclear power plants, mainly in Asia (China and India), but also in other newly industrialized countries around the world (Merkel and Schipek 2011). The high world market prices for fossil fuels and the growth of energy demand driven by continuing economic development, are strongly contributing for the rise of nuclear power, since high world market prices for fossil fuels have a great impact particularly in developing countries with low financial resources. It is estimated that about 450 nuclear plants are planned or under construction worldwide, which consequently raised the interest for the opening of new mines in many countries, such as France, UK, Argentina, Mexico and Mongolia, and has increased the uranium mining activity in the main uranium producing countries like Kazakhstan, Canada, Australia, Namibia, Niger, Russia, Uzbekistan and the USA (Brugge and Buchner 2011; Merkel and Schipek 2011). The World Nuclear Association predicts by 2015, world demands of about 77,000 tons of uranium, most of which coming directly from mines (Brugge and Buchner 2011). The increase in uranium mining worldwide will increase information and awareness of the epidemiological and toxicological effects of uranium, which will probably lead to controversy. So, it seems appropriate to raise some questions: i) Will the developing countries know how to deal with the legacy left by intense uranium mining activities? ii) Do these countries have appropriate legislation for the protection of the environment and populations living nearby? iii) Are they fully aware and informed about the risks posed by uranium mining? A negative answer to these questions will bring devastating consequences for populations, for the environment and ultimately to the world. Yet, other important questions are worth asking: i) Are we capable of fully control this type of energy? ii) Are we fully prepared for an effective response in the case of a nuclear disaster? I think that we all know the answer to these questions; this became clear with the recent nuclear disaster at Fukushima. The dangers associated with this nuclear renaissance demands for continued and more profound studies on the toxicity and epidemiology of uranium, since as researchers look deeper, many new effects are being found (Brugge and Buchner 2011). More studies on the impacts of uranium mines in the populations and

in the environment are also needed, to establish more effective measures of protection, enforcing the developing of new technologies for a safer extraction of uranium ore. Meanwhile, we are still dealing with the consequences of the uranium booms that occurred around the 50's and the 70's, as a result of a concerted effort to discover and exploit uranium resources to supply uranium for the production of military weapons and for the generation of nuclear energy, respectively (IAEA 2005). The end of these uranium rushes left behind a large number of abandoned uranium mines in several countries around the world, and Portugal was not an exception. About 60 deposits of uranium were exploited, in Portugal, during the 20th century, which caused various degrees of environmental impact according to the type of exploitation and the amount of radioactive ore extracted (Carvalho et al. 2005). Cunha baixa is one such example and constitutes the case study of this document. The main problems associated with this mine are due to heap leaching and i*n situ* leaching processes used for uranium extraction from pore ore, which has released metals and radionuclides to surface and groundwaters. Moreover, the tailing deposits around the mine pit, the deposition of sludge from the water treatment plant (rich in metals and radionuclides) and the irrigation of soil with contaminated water, also contributed for the contamination of soil and air. Bearing in mind all these contamination sources, it was considered a priority to study their impact in the wildlife and in the human populations living nearby.

The studies undertaken at the Cunha Baixa uranium mine, aimed to contribute with important information/knowledge on the chemical and radiological risks posed to indicator species (earthworms and small mammals), and also to the population living nearby. Another goal was to evaluate if earthworms and wood mice could be good indicators species for genotoxic effects, caused by the exposure to metal-rich and radioactive wastes of this uranium mine. Additionally, we intended to unravel and clarify the underlying mechanisms triggering responses to metals and radionuclides exposure, through the identification of potentially new molecular biomarkers. By studying the human population living nearby the abandoned mine, it was also intended to identify early warning signals of molecular and cellular damages, which could increase the risk of development of genetic diseases, as, for instance cancer. To accomplish these objectives several tasks were outlined, to evaluate the impact of uranium mining residues in inducing damages at different levels of biological organization in indicators species and in the human population. Genotoxic effects and increased levels of several contaminants were observed in all the organisms tested in this study, as well as in humans. Also, immunotoxic effects were observed in earthworms and humans, since, in both cases, changes in immune cell counts were observed, although in earthworms other effects were also observed.

Earthworms were chosen as test organisms, mainly due to their importance in the soil system and because they are widely used in standard ecotoxicological tests, to evaluate the potential adverse effects of chemicals on soil organisms. Small mammals such as rodents have shown to be sensitive to contaminants and appropriate bioindicator species for monitoring exposures and effects, thus the European wood mouse was also selected for this study.

Chapters 2 and 3, include experiments performed with earthworms exposed in the laboratory to contaminated soil from the Cunha Baixa uranium mine. Biomarkers such as DNA strand breaks, immune cell frequency and histological alterations were evaluated. The results revealed that exposure to these soil's contaminants, namely metals and radionuclides were able to induce genotoxic and cytotoxic effects, as well as to cause histological alterations and to inhibit growth, on this commonly used test species.

In chapters 4 and 5, earthworms were exposed *in situ* to contaminated soil from the Cunha Baixa uranium mine and also to the natural standard soil LUFA 2.2. Chambers were placed in a sludge deposition site near the mine pit and in a reference area. Results showed that *in situ* exposure to the contaminated soil caused DNA damages, changes in the earthworm's immune system, metals and radionuclides bioaccumulation, growth and reproduction inhibition. The exposure to these contaminants also induced changes in the transcriptome of exposed organisms, since the expression levels of several genes involved in important processes like metabolism, oxireductase activity, redox homeostasis, calcium homeostasis and response to stress showed alterations, comparing to those of the control organisms. The experiments undertaken in these chapters showed that the evaluation of genotoxicity and cytotoxicity endpoints can be combined with standard reproduction assays for the evaluation of the true risk of exposure to this type of contamination. The evaluation of the above referred endpoints will avoid a possible underestimation of the risks when only ecotoxicological parameters such as mortality, reproduction or growth are evaluated. More importantly, this study revealed that laboratorial assays, although lacking ecological significance, are important for the primary screening of the effects exerted by the contaminants, to characterize their toxicological impact under controlled environmental conditions. The experiments undertaken in these two chapters have given more realistic results, since *in situ* exposures characterize the true risks posed by the contaminants, taking into account the natural fluctuations of the environmental conditions, which in turn may influence the toxicological profile of the contaminants and also the response by target organisms. When comparing both laboratorial and *in situ* assays it was possible to observe that no significant differences were recorded, regarding the evaluation of DNA damages. However the opposite was

observed for immune cell counts and bioaccumulation, since immune cells behaved differently in the *in situ* assay when compared to the laboratorial assay, and also the bioaccumulation of contaminants was higher in the organisms exposed *in situ*. The importance and usefulness of gene expression analyses is reported in chapter 5. It allowed the characterization of the primary response in target organisms, which occurs at a very low level of biological organization, simultaneously allowing for the detection and development of new and more sensitive biomarkers of exposure that may be employed as early warning signals of the exposure to toxicants.

Chapter 6 reports a study performed in the European wood mouse, where our goal was to look up at the effects in animals living in the contaminated area. In this study, blood samples, collected from exposed and control animals (captured from a non-contaminated reference site), were tested for the presence of DNA strand breaks by comet assay, and have shown significantly higher DNA damages in the blood cells of animals living in the mine area. Additionally, single nucleotide polymorphisms (SNP's) and alterations of the expression levels of the tumor suppressor genes *Rb* and *P53* were determined by Real-Time qPCR in samples from the liver and kidney of the captured animals, and revealed SNP's in the *Rb* gene and alterations of the expression levels of *P53*. Moreover, to clarify cause-effects relationships, metals bioaccumulation was also evaluated in the liver, kidney and bones of the mice captured in both sites, revealing significantly higher levels of uranium and cadmium in the liver, kidneys and bones of mice captured in the mine area. These results suggest that the exposure to uranium mining wastes can cause genomic instability in the organisms exposed to them. Data collected from vertebrates living in/exposed to contaminated areas, are of utmost importance since they can bring pertinent information to be used in human risk assessment (Pereira et al. 2006).

Finally, in chapter 7, the potential genotoxic and immunotoxic effects caused by the exposure to uranium mining wastes in the population living close to the Cunha Baixa mine, were determined. This was accomplished through the collection of peripheral blood samples, from individuals of the Cunha Baixa village and from a reference population, that were tested for the presence of DNA strand breaks using the comet assay, for aberrant phenotype and immune cell count by flow cytometry and also for the presence of trace elements. Results showed that blood samples from individuals of Cunha Baixa presented higher levels of DNA damages, decreased cell counts of important immune cell populations, presence of uranium and manganese and decreased levels of zinc, when compared to individuals from the reference population. Overall the results showed that the exposure of the population of Cunha Baixa village to the mining wastes

(probably through the ingestion of contaminated food and water) has a negative impact on their health, since the genotoxic and immunotoxic damages herein described, as well as the presence of uranium and decreased levels of zinc in blood samples, may contribute to the vulnerability of these individuals to the development of genetic diseases such as cancer, considering the carcinogenic potential of the exposure to radioactive wastes and metals.

8.1.1 Highlights and major conclusions of this study

Considering the objectives of this thesis, we conclude that earthworms and wood mice are good indicator species for the detection of the genotoxic risk of exposure to metals and radionuclides. Moreover, the usefulness of toxicogenomic techniques in the clarification of the mechanisms underlying the responses of the organisms to the exposure to metals and radionuclides was also proved here, and was clearly shown that the response to environmental stress involves many molecular networks that can only be studied through the application of such techniques. The study of the human population living nearby the abandoned mine, allowed the detection of early warning signals of molecular and cellular damages, such as the presence of DNA strand breaks and also the decrease of important immune cell populations like, Natural Killer cells and T Lymphocytes in the exposed population. These results show that an immediate intervention is required, to minimize the exposure and potential damaging effects on wildlife and human populations living nearby this area, once damaging effects at a molecular and cellular level, may potentially cause genomic instability and increased risk of developing genetic diseases. The information collected by means of a questionnaire, helped us to rule out the presence of other factors that could confound the results and also to identify potential contamination sources.

The major conclusions to be withdrawn from this study are highlighted bellow:

- Soils from abandoned uranium mining areas pose serious risks to the overall fitness and survival of epigeic earthworm's populations and communities, by affecting growth and reproduction and also by inducing genotoxic and cytotoxic effects in these organisms.
- The evaluation of DNA strand breakage, immune cells frequency in coelomocytes and histological changes in earthworm's body wall and gastrointestinal tract, can be used as reliable and sensitive biomarkers of harmful effects yielded by available metals and radionuclides. As so, they are important endpoints to be used in the earthworm reproduction

assay, to evaluate the sub-lethal toxicity of soils compromised by the deposition of similar waste materials

- The evaluation of bioaccumulation, genotoxicity, cytotoxicity and histological biomarkers, along with other parameters at the individual level in standard reproduction assays, conducted in the laboratory or *in situ,* could help elucidating the mechanisms of toxicity, like growth and reproduction impairments. Therefore, helping to reduce the uncertainty of the information provided by ecotoxicological tests in the risk assessment process of contaminated areas.
- The use of SSH technique provided a global perspective of the gene expression profile of earthworms exposed to metals and radionuclides, highlighting the complexity of the responses to the mixture of these inorganic stressors, under environmental conditions. Likewise, gene expression studies provide indications at the molecular level of the effects of the exposure to toxicants, but most importantly, to the development of more sensitive and specific biomarkers that may be employed as early warning signals of exposure to toxicants
- Small mammals living nearby uranium mines, have a greater risk of suffering DNA damages and therefore of developing serious genetic disorders, since the exposure to uranium mining wastes caused significant DNA damages and alterations in the expression of *p53* tumor suppressor gene. This may have serious and unpredictable impacts on exposed organisms.
- Comet assay and flow cytometry proved to be the techniques appropriate for being coupled with laboratorial and *in situ* assays, to evaluate the toxicity of contaminants such as metals and radionuclides in earthworms and wood mice. The comet assay is a relatively cheap technique that is very easy to be applied in routine evaluations and that gave concordant results in all the species here analyzed.
- Human populations, inhabiting nearby abandoned mines that were explored in the past for radioactive metal ores, are at risk, since genotoxic effects and immunotoxic effects were detected in the population of the Cunha Baixa village, which potentially may compromise the genetic integrity of germ cells and their defenses against cancer development.
- Immediate intervention is needed to minimize further negative consequences on humans and wildlife to uranium mining wastes.

8.2 Future perspectives

Many questions are still to be answered regarding the effects of the exposure to wastes resulting from the extraction of radioactive ores and also the consequences of such extraction activities in the surrounding environment. Since uranium extraction activities are occurring in several parts of the world, further knowledge is needed to effectively determine the actual risks to animals and human populations living nearby these areas, in order to take the appropriate measures to minimize the exposure risk and all the potentially negative effects associated with it.

Some aspects requiring further investigation are below summarized:

- A detailed analysis in somatic cells and in germ cells of the DNA repair systems, gene expression profiles and also the presence of mutations in important genes, such as oncogenes and genes involved in DNA repair, in earthworms, small mammals and humans, will contribute for a better understanding of the mechanisms involved in metals and radionuclides toxicity and also for the identification and validation of new genetic biomarkers.
- A more profound study regarding the responses and effects of radiation in the immune system of earthworms, small mammals and humans and also regarding specifically the type of DNA damages caused by the exposure to metals and radionuclides.
- Determine the target organs for radionuclides accumulation in earthworms. That information would give an insight of which organs are the most affected by the exposure to these contaminants.
- It will also be important to test earthworms genetic responses to serial dilutions of the contaminated soil to determine which dilution factor should be used in future remediation actions in abandoned uranium mines.
- To study the influence of the ingestion of local products and water, in the development of genetic damages in domestic animals.
- Studies in other former mining sites will provide a more clear and accurate scenario of the real problem of abandoned uranium mines and its associated residues.
- Finally, an attempted to establish a parallelism between the effects observed in earthworms, mice and humans.

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